The Role of Dispersal and Adaptive Divergence in the Diversification and Speciation of the Tribe Brassiceae and Genus *Cakile*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Adaptation is central to our understanding of the origin of biological diversity. Yet whether adaptive divergence promotes the formation of new lineages remains poorly understood. My dissertation addresses the role of adaptive divergence in diversification and speciation. I also investigate an alternative mechanism: dispersal, which can promote diversification and speciation through its effects on gene flow and allopatry. To address the role of divergent adaptation and dispersal in the process of diversification, I combine comparative methods with quantitative genetics to characterize patterns of diversification and speciation in the tribe Brassiceae and genus *Cakile*. I start with a comparative study of the role of dispersal and adaptation in diversification, and then focus on the role of climatic and latitudinal divergence in the processes of adaptive divergence and speciation. In general, I find limited evidence for the role of divergent adaptation in the evolution of intrinsic reproductive isolation. Diversification in the tribe Brassiceae appears to be mediated by dispersal ability, while in the genus *Cakile*, the evolution of intrinsic reproductive isolation is largely independent of ecological divergence. Thus, while divergent adaptation to novel habitats and climate are likely occurring in Brassiceae, mediated in part by the evolution of long-distance dispersal, the evolution of intrinsic genic reproductive barriers appears to not be influenced by adaptation.

Dedication

I would like to dedicate my dissertation to my parents, Ann and Steve Willis. Without their support and encouragement the probability of all of these words and figures coalescing into something readable would be slim to none. I would also like to dedicate my dissertation to my grandfather, George Willis. It was you who got me caught up in this demandable thing they call science, and I'll never forget it.

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Drs. Jocelyn Hall, Elena Kramer and Kathleen Donohue contributed extensively to Chapter 2, and will be co-authors on a published manuscript. Jocelyn Hall, in particular contributed both molecular and phenotypic data used in the analyzes of Chapter 2. Elena Kramer provided advice on the phylogenetic analysis and contributed to the conceptual development of this project. Kathleen Donohue contributed to conceptual development of the project, provided resources for its execution, and provided commentary on the analysis and writing. In addition, Duke University undergraduate Timothy Wang was instrumental in the collection of the molecular data used in the phylogenetic analyzes, and will appear as a co-author on the manuscript. Dr. Rafael Rubio de Casas also contributed conceptual and analytical advice to the project. And, finally, Jamie Sabo, a high-school student, collected a significant proportion of the fruit and seed morphology data.

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1. Introduction: An Overview

Adaptation is central to our understanding of the origin of biological diversity. The theories adaptation and evolution were born from Darwin's and Wallace's attempt to explain the diversity—and its re-occurring patterns—they observed across the globe. In their minds, it was adaptation that was the engine of diversity.

From the time of Darwin, two general approaches have been employed to address the role of adaptation in diversification. First, the comparative approach focused on identifying associations between morphological or behavioral diversity and ecological diversity of species or higher taxonomic levels (Endler 1986). The most famous example, of course, is work with the Galapagos finches, where generations of work, dating back to Darwin, have found that the diversity of the group, centered on variation in beak shape, was driven by adaptation to different food sources (Darwin 1859; Grant 1999; Donohue 2011). The Galapagos finches were one of the first notable examples of an adaptive radiation. An adaptive radiation refers to a group of closely related species that have rapidly diversified across a range of distinct ecological niches(Givnish and Sytsma 2000; Schluter 2001). Many similar examples of adaptive radiations have since been identified (Gavrilets and Losos 2009).

The second approach to understand the role of adaptation in diversification has focused on the micro-evolutionary, or population-level, mechanisms that underlie the process of a single lineage splitting into two evolutionarily independent lineages. This field of speciation biology originates from work in the broader field of population genetics (Coyne and Orr 2004; Nosil 2012). From the population-genetics perspective, species are defined by their evolutionary independence; that is to say, by the degree to which they do not exchange genetic information. This definition of species, known as the Biological Species Concept, emphasizes barriers to gene flow between species (Dobzhansky 1937; Mayr 1942; Coyne and Orr 2004), and studies of the population dynamics of speciation therefore focuses on the evolution of reproductive barriers (Coyne and Orr 2004; Rieseberg and Willis 2007). Discerning the relationship between population processes that account for the evolution of reproductive isolation and macro-evolutionary patterns of diversification within clades is a major challenge in the field of evolutionary biology (Schluter 1996; Arnold et al. 2001; Steppan et al. 2002; Cavender-Bares and Wilczek 2003; Butlin et al. 2009).

Reproductive barriers are mechanisms that prevent gene flow between populations, and thus permit these populations to evolve independently of one another. Reproductive barriers are diverse and range from spatial or temporal (e.g., phenological) separation between species that limit interactions, to genetic

incompatibilities that are manifest as inviable or infertile hybrid offspring. The role of adaptive divergence in the evolution of reproductive barriers has been, at times, controversial (Coyne and Orr 2004; Levin 2004a; Mallet 2008; Schemske 2010; Sobel et al. 2010). The controversy is not whether adaptation contributes to their evolution *per se*, but rather concerns the extent to which it contributes to specific types of reproductive barriers, the extent to which specific reproductive barriers contribute to the process of speciation, and thereby whether ecological adaptation accelerates the speciation process itself. Of particular controversy is the extent to which adaptation contributes to the evolution of "intrinsic" reproductive barriers, which are not under direct ecological selection but instead caused by intrinsic genic incompatibilities that lower fitness regardless of the ecological environment (Schluter and Conte 2009).

Natural selection can be divergent (i.e., selection for different traits in populations inhabiting different locations) or parallel (i.e., selection on the same traits in both populations). Divergent versus parallel selection are predicted to contribute to the evolution of reproductive isolation differently (Langerhans and Riesch 2013). Divergent selection is expected to promote the evolution of reproductive barriers that are involved with specific ecological functions in contrasting environments. These include several pre-zygotic barriers, such as pollinator isolation and ecological isolation acting through immigrant inviability

(Darwin 1859; Nosil and Mooers 2005; Rundle et al. 2005; Hendry et al. 2007; Nosil et al. 2009; Via 2009). These also include disrupted adaptation of hybrids. Collectively, these are referred to as extrinsic post-zygotic barriers (Dobzhansky 1937; Mayr 1942; Coyne and Orr 2004; Levin 2004a; Rundle and Nosil 2005; Nosil et al. 2009). In contrast, parallel adaptation is not expected to contribute to extrinsic reproductive barriers via ecological isolation or disrupted adaptation. However, if adaptation to similar environments occurred through different pathways (Manceau et al. 2010; Smith and Rausher 2011; Thurber et al. 2013), phenotypic and genetic incompatibilities may be manifest in hybrid offspring (Schluter 2009; Nosil and Flaxman 2010; Feder et al. 2011; Langerhans and Riesch 2013).

Intrinsic reproductive barriers, in contrast to extrinsic barriers, arise through intrinsic genic incompatibilities in zygotes. Whether divergent selection accelerates the evolution of post-mating barriers such as pollen-pistil incompatibilities or barriers that reduce hybrid fitness through intrinsic incompatibilities (i.e., intrinsic post-zygotic barriers) is less clear. Theoretical models predict that intrinsic post-zygotic barriers will evolve at similar rates under divergent and parallel selection (Barton 2001; Gavrilets 2004; Unckless and Orr 2009; Conte and Arnegard 2012) provided the alleles that contribute to adaptation are unique to each population. However, intrinsic post-zygotic

barriers would evolve more slowly under parallel selection if the same alleles were fixed in both populations, as would be the case if the number of new alleles were limited or if selection operated on similar pools of standing variation (Coyne and Orr 2004; Barrett and Schluter 2008; Schluter 2009; Schluter and Conte 2009). Thus, when unique, new mutations are not the only source of genetic variation for adaptation, intrinsic post-zygotic reproductive isolating barriers are generally thought to evolve more quickly under divergent than parallel selection. In theory, these same predictions could be extended to the evolution of post-mating pre-zygotic barriers, (e.g. pollen-pistil incompatibilities) under divergent versus parallel selection.

Ecological adaptation would contribute to the evolution of intrinsic reproductive barriers if loci underlying intrinsic genic incompatibilities either were pleiotropically or physically linked to loci under direct selection (Coyne and Orr 2004; Rundle and Nosil 2005). A small, but growing, number of genes causally implicated in intrinsic genic incompatibilities have been conclusively identified, and they generally show mixed evidence of having contributed to adaptive divergence (Rieseberg and Blackman 2010; Wolf et al. 2010; Nosil and Schluter 2011), though some have been found to exhibit genetic signatures of positive selection (Presgraves et al. 2003; Tang and Presgraves 2009). Even fewer genes have been shown to be linked to loci under divergent selection (Bomblies

and Weigel 2007; Wright et al. 2013). This dearth of empirical evidence has led to skepticism that ecological adaptation contributes appreciably to the evolution of intrinsic reproductive barriers (Coyne and Orr 2004). Perhaps somewhat less controversial is the possibility that ecologically divergent loci involved in mating interactions might also pleiotropically contribute to post-mating, prezygotic incompatibilities; for example, loci contributing to adaptation to specific pollinators can alter style length and pollen size in ways that would affect pollen tube growth in divergent styles (Delph et al. 1997; Travers 1999; Ruane and Donohue 2007). Thus few empirical examples conclusively demonstrate direct associations between loci responsible for ecological adaptation and those contributing to intrinsic reproductive barriers, either via pleiotropy or linkage. Admittedly, however, few studies to date have identified genes directly involved in genic incompatibilities, or even genes directly involved in ecological adaptation, especially in the same system.

In addition to adaptive divergence, the other major population process that contributes to reproductive isolation is allopatry itself: the spatial isolation of populations that prevents gene flow among them. Allopatry directly leads to genetic independence and therefore also influences the dynamics of adaptive divergence. Thus, there has also been great interest in the role of allopatric isolation in the diversification of taxa (Mayr 1942; Stebbins 1974; Barraclough and

Vogler 2000). In the now classic review of speciation biology, Coyne and Orr (2004) state that the geographical context of speciation is among the most contentious issues within the field (Coyne 2007; Butlin et al. 2008; Bird et al. 2012). Allopatry results in immediate reproductive isolation (i.e., zero gene flow), which in turn allows isolated populations to evolve independently. Under these conditions populations are more likely to diverge, even to the extent that they could become reproductively isolated after secondary contact (Coyne and Orr 2004).

Allopatry can occur either by vicariance—via changes in physical geography or by dispersal—determined by biological attributes of organisms and how they interact with dispersal vectors. Dispersal, as a property of the organism can evolve (McPeek and Holt 1992; Olivieri et al. 1995; Levin et al. 2003; Cousens et al. 2008; de Casas et al. 2012). Depending on its magnitude, dispersal can either promote or limit gene flow (Birand et al. 2012). For instance, dispersal could decrease speciation rates by limiting population fragmentation and adaptive divergence, and decrease extinction rates by expanding range size and ecological niches (through niche expansion). Alternatively, long-distance dispersal could cause populations to be isolated from other populations. The relative balance between these two processes could influence the overall diversification rate. As such, dispersal is a central process that influences the

degree of allopatry and therefore the probability of diversification of taxa. Thus, the evolution of dispersal-related traits and dispersal ability can influence patterns of diversification independent of adaptation *per se*. The relationship between dispersal ability and diversification is therefore important to determine.

My dissertation addresses the role of dispersal and adaptive divergence in diversification and speciation. To more fully understand how the process of speciation and divergent adaptation results in diversification, I take an integrated approach, combining both comparative methods with quantitative genetics to characterize patterns of diversification and speciation in the tribe Brassiceae. I start with a comparative study of the role of dispersal and adaptation in diversification, and then focus on the role of climatic and latitudinal divergence in the processes of adaptive divergence and speciation.

I explore these questions in the plant tribe Brassiceae (Brassicaceae), home to several familiar domestic crops such rape seed and cauliflower (Warwick et al. 2010). The tribe is also home to the genus *Cakile* (Rodman 1974), which will be the focus of several chapters of this dissertation. The Brassiceae exhibit a diversity of fruit morphology associated with both limited and long-distance dispersal (Gomez-Campo 1980; Al-Shehbaz 1985; Hall et al. 2011). This makes the system ideal for studying the evolutionary implications of dispersal ability both across species (Hall et al. 2011) and within species level (Payne and Maun 1981;

Donohue 1997; 1998a; Westberg and Kadereit 2009). The tribe also inhabits a wide variety of habitat types and is distributed across a wide climatic and latitudinal range. The center of diversity of Brassiceae is in the southwestern Mediterranean region. However, the tribe ranges through western Asia to India and Africa, with a few species found in the Americas (Gomez-Campo 1980; Al-Shehbaz 1985). The Brassiceae also exhibit a wide range of habitat types including desert, ruderal, field and coastal habitats. This diversity in habitat is accompanied by diversity in climate, with species occurring from the Arctic Circle to the Saharan Desert. The variation in the geographic range of species in the tribe is also extensive. While most species are endemic to small regions of the western Mediterranean, some species range across the entirety of Europe.

Within the tribe, the genus *Cakile* is notable for the degree of variation in climate and range that it inhabits. All but one of the seven species in the genus grow along the coastal strand (Rodman 1974). The genus has been used to study the demographics and evolutionary ecology of strand plants (Barbour 1970; Rodman 1974; Keddy 1982; Donohue 1997; 1998b; Debez et al. 2004). Given its reproductive ecology and propensity for dense offspring populations, it has also been used to study the maternal effects of density (Donohue 1998b) and kinship recognition (Dudley and File 2007). While limited to the coastal strand, *Cakile* has nonetheless diversified across a while range of latitudes from Iceland to the

coasts of the Mediterranean and Caribbean. Furthermore, species in both North America and Europe have undergone apparent divergence along similar latitudinal and climatic gradients (Appendix A), as well as undergone divergence within southern climates. This makes the genus ideal for studying the role of climatic factors in divergent adaptation. Furthermore, given the multiple apparent parallel adaptation events in latitude and climate, the genus offers the opportunity to compare the influence of divergent versus parallel adaptation on the evolution of reproductive isolation. In my dissertation, I use the Brassiceae and the *Cakile* clade in particular to explore the role of dispersal and adaptive divergence in diversification and speciation.

In Chapter 2, I investigate the evolution of dispersal ability and its impact on adaptation, geographic range, and diversification in the tribe Brassiceae. This tribe thus offers a unique opportunity to examine the evolution of dispersal-related fruit traits, relationships between fruit and seed traits, their associations with ecological factors such as habitat, climate, range size and lineage diversification. More specifically, I use a comparative approach to address the following questions: 1) Do dispersal-related fruit traits exhibit correlated evolution? 2) Are the evolution of dispersal-related fruit traits correlated with the evolution of seed traits? 3) Is the evolution of dispersal-related fruit traits

associated with changes in range size, habitat, or climatic niche? 4) Do transitions in dispersal ability or habitat influence diversification rates?

Results from Chapter 2 suggest that a shift to a coastal habitat, primarily in the genus *Cakile,* both increased diversification via speciation rates and facilitated the expansion of latitudinal range. In Chapter 3, I investigate adaptations to climatic factors in the genus *Cakile* along a latitudinal gradient. The genus *Cakile* has diversified repeatedly across a wide-range of seasonal environments and latitudes. Furthermore, there are recognized morphological and genetic differences between latitudinal races that suggest adaptive divergence may have occurred along a seasonal cline (Rodman 1976; Clausing et al. 2000; Gormally and Donovan 2011). To test this prediction, I use a simulated reciprocal transplant experiment to test for adaptive divergence in two species of *Cakile* that have both undergone parallel morphological and genetic divergence along a latitudinal gradient. The experiment was performed in growth chambers that simulated differences in key climatic factors across latitude, by manipulating changes in temperature and day-length over the course of a growing season for four sites along the full range of the two species. We tested for genetic divergence in key life-history traits and tested for latitudinal adaptation using measures of fitness.

Along with additional evidence outlined in the Appendix A, Chapter 3 suggests that *Cakile* has undergone adaptation along a climatic and latitudinal gradient. In Chapter 4, I investigate whether ecological divergence along a climatic gradient promoted the evolution of reproductive isolation barriers in *Cakile*. To address this question, I characterize the evolution of intrinsic (post-mating pre-zygotic and post-zygotic) reproductive isolation across the genus *Cakile* and the allied genus *Erucaria* and tested whether the intensity of reproductive isolation between taxa was correlated with their ecological divergence. Using a comparative approach, involving 18 taxa, I addressed the following questions: 1) Which components of intrinsic post-zygotic reproductive isolation are strongest and have evolved fastest? 2) Do these components of intrinsic post-zygotic reproductive isolation exhibit linear or non-linear rates of evolution? 3) Does ecological divergence correlate with levels of intrinsic post-zygotic reproductive isolation, after accounting for genetic distance?

In Chapter 5, I again test whether ecological divergence promoted the evolution of reproductive isolation, but more precisely control for genetic distance and ecological divergence using a focal-cross design. A focal-cross design compares the level of reproductive isolation between two sister taxa and a focal taxon, thus controlling for genetic distance. By using sister taxa with similar versus divergent ecological characteristics relative to the focal taxon, I can

explicitly test the effect of ecological divergence on the level of reproductive isolation. In this chapter, I test the effect of latitudinal divergence on the evolution of post-mating pre-zygotic and intrinsic post-zygotic reproductive isolation in the genus *Cakile* using two pairs of divergent sister species, *C. edentula* (North America) and *C. maritima* (Europe).

2. The Evolution of Dispersal and Diversification in the tribe Brassiceae (Brassicaceae)

2.1 Introduction

Dispersal is one of the primary factors that influence evolutionary rates and outcomes, and it has been recognized as such since the earliest days of theoretical population genetics (Wright 1931). Dispersal ability and establishment success influence several major population processes including isolation, adaptive divergence, and extinction probability (Levin and Kerster 1975; Howe and Smallwood 1982; Willson and Traveset 2000; Benton and Bowler 2012). The influence of dispersal on macro-evolutionary processes, however, is less well understood. Theoretically, by influencing these population dynamics, dispersal can also influence macro-evolutionary patterns of species distributions and diversification. Here we investigate how traits associated with dispersal and establishment influence geographic range, habitat, and diversification rates.

Attributes of the dispersal propagule—in plants, either seeds, fruits, or more rarely vegetative segments (de Casas et al. 2012); in animals, gravid females or dispersing groups or individuals (Greenwood 1980; Bowler and Benton 2005)—influence the pattern and distance organisms are dispersed. The pattern and distance of dispersal can in turn influence gene flow and demography in ways that can impact adaptation, population size, and population isolation (Levin and Kerster 1975; Howe and Smallwood 1982; Slatkin 1985; Dieckmann et

al. 1999). Modifications to dispersal-related traits and their impact on population dynamics can in principle have subsequent large-scale ecological and evolutionary consequences.

Limited dispersal is predicted to limit gene flow between populations, promoting population fragmentation, genetic divergence, local adaptation, and endemism (Levin et al. 2003). Efficient dispersal, in contrast, is predicted to increase gene flow, inhibiting population fragmentation and local adaptation (Cain et al. 2000). Long-distance dispersal may increase the probability of encountering novel habitats and facilitate adaptation along range boundaries by providing genetic variation and maintaining population size. If it is extreme enough, long-distance dispersal may actually promote isolation through long-distance colonization events and promote shifts in habitat or niche (Cain et al. 2000; Levin et al. 2003).

Effects of dispersal on colonization and establishment can in turn influence range size. Species with limited dispersal are hypothesized to have smaller ranges, while species with long-distance dispersal are predicted to have larger ranges (Holt 2003; Gaston 2003; Lester et al. 2007; Hubbell 2008). Empirical studies of the effect of dispersal ability on range size however are mixed, with several studies finding little to no evidence for these expected relationships at all (Edwards and Westoby 1996; Lester et al. 2007; Gove et al. 2009).

The meta-effects of dispersal ability on range size and ecological divergence are likely to have macro-evolutionary effects on diversification rates. Diversification is the net result of speciation and extinction (Ricklefs 2007). Both of these processes are likely to be influenced by changes in dispersal ability (Birand et al. 2012). For instance, limited dispersal is predicted to increase the rate of speciation by promoting population fragmentation and adaptive divergence. However, limited dispersal is also expected to increase the likelihood of extinction by promoting decreased range size and endemism. In contrast, long-distance dispersal could decrease speciation rates by limiting population fragmentation and adaptive or genetic divergence, and decrease extinction rates by expanding range size and ecological niches (through niche expansion). Alternatively, long-distance dispersal could promote speciation via rare long-distance colonization events that impose allopatry.

In plants, evidence for the role of dispersal in diversification as been limited to systems with animal dispersed fruits. Lengyel et al. (2009) found that the evolution of long-distance dispersal via ants (myrmecochory) was associated with increased diversification rates in several angiosperm lineages, yet they were unable to determine whether these patterns were caused by increased speciation or decreased extinction rates. Similarly, the evolution of fleshy fruits, a character associated with long-distance dispersal by frugivores, has been found to be

associated with increased rates of diversification in several plant groups (Givnish 2010). For instance, Tiffney and Mazer (1995) identified an association between taxonomic richness and the presence of vertebrate dispersal across the angiosperms, and Moore and Donohgue (2007) identified a significant association between fleshy fruits and increased diversification rates in the Dipsacales.

There is a long history in plant biology of interpreting morphological traits of fruits in terms of how they influence seed dispersal (Cousens et al. 2008; de Casas et al. 2012). Traditionally ascribed "dispersal syndromes" are based on presumed functional aspects of fruit morphology that determine dispersal vectors (van der Pijl 1982; Tiffney 1984; Murray 1986). These dispersal syndromes are often interpreted in terms of the likelihood or distance of dispersal (Gautier-Hion et al. 1985; Hamrick and Loveless 1986; Mandák and Pyšek 2001; Lomáscolo et al. 2010). For example, seeds in fleshy fruits dispersed by birds (ornithochory) are considered to have long-distance dispersal, while seeds in fruits that develop underground (geocarpy) are considered to have limited dispersal.

Specific fruit features have been shown to directly influence dispersal ability. In non-fleshy fruits, whether or not fruits dehisce is a major determinant of dispersal. Dehiscence is a process whereby the valves surrounding mature

seeds detach from the separation layer, thereby exposing and releasing seeds (Meakin and Roberts 1990b; Ferrándiz 2002; Roberts et al. 2002). Indehiscence occurs in many taxa, such that seeds are enclosed within the valves, preventing seeds from being dispersed independently from one another. Indehiscence can prevent dispersal from the maternal parent altogether, as evident from successful agricultural breeding programs against "seed shattering" (Meakin and Roberts 1990a,b; Spence et al. 1996; Ferrándiz 2002; Roberts et al. 2002). In indehiscent fruits, pericarp features can influence the ability of seeds to travel though air, water, or on fur and thereby influence dispersal distance (Murray 1986; Bremer and Eriksson 1992; Cousens et al. 2008). For example, buoyancy has been shown to by enhanced by wings, hairs, or corky texture (Payne and Maun 1981; Kubitzki and Ziburski 1994), while travel through air is enhanced by wings that lower wing-loading ratios (Augspurger 1986; Tackenberg et al. 2003).

Fruit traits may also evolve in response to factors unrelated to dispersal, but in ways that may indirectly influence dispersal ability. For example, the evolution of indehiscence may evolve as a mechanism to protect seeds from desiccation in dry environments (Ellner and Shmida 1981; Gutterman 1994), but can also lead to limited dispersal. Thus adaptation of fruit and seed traits to specific environmental conditions can have indirect effects on dispersal ability.

Dispersal-related traits may co-evolve. For example, selection on a trait that indirectly limits dispersal might result in the evolution of another trait that promotes dispersal, thereby compensating for the previous dispersal restriction. Such correlational selection can be difficult to detect within species, since its detection relies on genetic variation in both traits still being present. However, comparative approaches are valuable for detecting evidence of such correlational selection, because it can be reflected in macro-evolutionary patterns of correlated trait changes across whole phylogenies (Ackerly 2000).

Fruit traits may also coevolve with seed traits. In particular, seed size has been expected to coevolve with dispersal ability (Tiffney 1984). First, seeds dispersed long distances to uncertain environments may be expected to be more highly provisioned to increase establishment success after dispersal (Cain et al. 2000). Conversely, larger seeds may have reduced dispersal because of functional constraints, especially in gravity or passively dispersed propagules (Guo et al. 2000; Leishman et al. 2000). Bet-hedging may also favor widely dispersed propagules that are small, thereby maximizing the probability that at least some propagules land in suitable environments (Mandák and Pyšek 2001). Dispersal attributes may coevolve not only with seed size, but also with the number of seeds contained within a dispersal propagule. For example, the evolution of an indehiscent pericarp results in all seeds within a fruit being dispersed as a single

unit, which would influence the dynamics of post-dispersal competition and impose selection to reduce sibling competition; such selection could, in turn, result in a reduction in the number of seeds per fruit (Imbert 2002).

In sum, dispersal ability can evolve through the evolution of known traits of fruits, and these traits can evolve with various degrees of independence from each other and from seed traits. Knowing the extent to which these traits evolve independently, and the extent to which their evolution is associated with range, habitat, and diversification rates would provide insight into how dispersal influences macro-evolutionary patterns of adaptation and diversification.

The tribe Brassiceae (Brassicaceae) exhibits much diversity of fruit morphology that corresponds to distinct dispersal modes. It also exhibits diversity in the size of geographic range, habitats, and climates (Gomez-Campo 1980; Al-Shehbaz 1985; Warwick et al. 2010). Brassiceae, therefore, offers an opportunity to examine the evolution of dispersal traits and their associations with seed traits, range size, niche shifts and diversification rates.

The tribe Brassiceae exhibits four major morphological fruit traits that correspond to dispersal ability: indehiscence, the joint, the abscission zone on the joint, and pericarp features (Rodman 1974; Donohue 1998a; Hall et al. 2011). *Indehiscence* is the absence of a dehiscence zone typical of Brassicaceous siliques, which results in the fusion of the valves/pericarp. With indehiscence, seeds are

not released after maturation, but enclosed within the fruit, which acts as the dispersal propagule (Hall et al. 2006). A *joint* separates the fruit into separate proximal and distal segments (this is termed heteroarthrocarpy). The joint appears to be the ancestral juncture between valves and stigma, but in heteroarthrocarpic fruits, the placental tissue (the replum) protrudes into the stigmatic tissue and enables seeds to be matured above the valves (Hall et al. 2006; 2011). Typically, only one or two seeds are present above the joint. Joints can be accompanied by the presence of an *abscission zone* that permits the distal segment (the segment above the valves) to detach. For taxa with a joint, the distal segment is always indehiscent (being surrounded by ancestrally stigmatic tissue, which never had a dehiscence zone), while indehiscence in the proximal segment varies among taxa, presumably according to whether they have lost a functional dehiscence zone. When joints can abscise, distal segments can be dispersed from the maternal parent, but indehiscent proximal segments frequently remain attached to the maternal parent even through germination (Donohue 1998b). The most common pericarp feature in the tribe (~60% of taxa with a pericarp feature) is corky pericarps, which promote flotation and dispersal by water (Rodman 1976; Payne and Maun 1981).

These traits are not ubiquitous across the tribe, nor is it clear how tightly associated they are (Gomez-Campo 1980; Hall et al. 2011). Approximately 40% of

described genera have heteroarthrocarpic fruits, i.e., have fruit with a joint. Within the subset of heteroarthrocarpic genera, only ~60% of genera have an abscission zone (Gomez-Campo 1980). Similarly, ~60% of genera with a joint exhibit indehiscence of proximal fruits, while a small number (~4%) of genera without a joint exhibit indehiscence. Additionally, ~25% of genera have a clearly definable pericarp feature. These traits have evolved several times independently (Hall et al. 2011), providing evolutionarily independent events to test how these traits have co-evolved and whether they are associated with shifts in niche, range, or diversification rates.

These four fruit traits is likely to impact dispersal ability in Brassiceae in several ways. Indehiscence could reduce dispersal by restricting the dispersal of independent seeds (Lu et al. 2010). Alternatively, indehiscence could promote dispersal if accompanied by dispersal-promoting pericarp features. A joint also is expected to reduce dispersal because it is accompanied by the evolution of an indehiscent distal segment. However, the evolution of an abscission zone in conjunction with a joint is likely to increase dispersal as it allows for seeds in the detached segment to be dispersed as a protected propagule (Imbert 2002). The evolution of pericarp features is also expected to promote dispersal (Gautier-Hion et al. 1985). Finally, the evolution of indehiscence and a joint both result in the containment of seeds within the pericarp and therefore my accompany

changes in seed size and seed number per fruit. These changes could also affect dispersal ability and post-dispersal success.

The diversity of fruit morphology associated with dispersal ability in the tribe Brassiceae offers a unique system with which to examine the co-evolution of dispersal-related fruit traits, relationships between fruit and seed traits, their associations with ecological factors such as range size and habitat, and macroevolutionary patterns of diversification. To understand the evolution of dispersal ability and its impact on subsequent shifts in range, niche and diversification, we address the following questions: 1) Do the major dispersal-related fruit characteristics in Brassiceae exhibit correlated evolution? 2) Is the evolution of seed traits correlated with the evolution of fruit traits? 3) Is the evolution of dispersal-related fruit traits and seed traits associated with changes in range size and with shifts habitat or climatic niche? 4) Does the evolution of dispersal-related fruit traits or transitions in habitat effect diversification rates?

2.2 Material and Methods

Taxon sampling. Samples were included from across the Brassiceae and represented 64% of recognized genera and 26% of recognized species based on Warwick et al. (2010; Table 1). Taxonomic sampling for the Brassiceae was similar to that of Hall et al. (2011; see Hall et al. 2011 for voucher information). Taxonomic sampling was expanded from Hall et al. (2011) primarily within the

genus *Cakile*, with thirteen additional species, subspecies, and populations included in the phylogenetic analysis. The heavy sampling of *Cakile* was primarily for use in additional comparative studies within the genus (see Chapter 4). For this study, subspecific taxa were not included in the final analyzes. Two additional species of *Brassica* (*B. juncea* and *B. rapa*) were added based on data available through NCBI—GenBank (www.ncbi.nlm.nih.gov/genbank). Two additional outgroups were also included (*Arabidopsis thaliana* and *Isastis tinctoria*) based on data available through NCBI—GenBank. Leaf material for DNA extractions was obtained from both field collections and plants grown in the greenhouse from seed stock. The majority of non-*Cakile* species were obtained from the specialized Brassicaceae seed bank at la Universidad Politecnica de Madrid, Spain. Field collections were conducted along the east coast of the United States, the Great Lakes, and throughout the Caribbean from 2004 to 2010. Plants from both the seed stocks and field were grown in Research Greenhouses at Duke University (Durham, NC) prior to leaf collection.

DNA extraction and selection of molecular markers. Total DNA was isolated from fresh leaf material or silica-dried leaves using Plant DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) and standard CTAB protocols (Doyle and Doyle 1987).

Four markers, two nuclear (*ITS1-ITS4, Fnr*) and two chloroplast (*psbA-trnH, Bras4-trnG*), were sampled for each taxon. These four markers were sampled in addition two markers (nrDNA: *phyA* and cpDNA: *matK)* previously sequenced for the same set of taxa by Hall et al. (2011).

Low-copy nuclear markers often exhibit higher rates of evolution and can be more informative, particularly among recently divergent taxa. However, nuclear markers may also obfuscate resolution because of past hybridization and polyloidization events (Warwick and Hall 2009). In contrast, while the chloroplast genome typically evolves at slower rate, it is not subject to the complications of hybridization and polyploidy (Wendel and Doyle 1998). Because of their different evolutionary histories, the chloroplast and nuclear genomes are likely to result in different phylogenetic hypotheses for given clade. In order to capture the potential variation in phylogenetic resolution across genomes, we sampled markers from both.

DNA amplification, sequencing and alignment. Amplification and sequencing primers for all regions were adapted from previous studies (Chase et al. 2007; Parisod and Besnard 2007; Shaw et al. 2007; Song and Mitchell-Olds 2007; Hall et al. 2011). PCR reactions for all regions used the mix: 10 μL ddH2O, 1 μL template DNA, 1 μL Econo- *Taq*, (Lucigen, Cat. No. 30033-0), dNTPS, buffer, 1μL forward primer, and 1μL reverse primer. Regions were amplified in 20 μL with an

Eppendorf, Master Cycler epigradient S thermal cycler using an initial 5 min. denaturation at 80°C follow by 30 cycles of 95°C denaturation for 1 min., 1 min. annealing at 50°C, and 4 min. extension at 65°C; followed by 5 min. of final extension at 65°C. PCR products were cleaned using a PCR Purification Kit (Invitrogen K3100-01 Carlsbad, California, USA).

Nuclear regions were subsequently cloned for a subset of taxa to identify multiple copies using Quigen Cloning Kit. For *Fnr*, copy-specific primers were designed to eliminate the need for further cloning. For *ITS,* all samples were cloned.

Cycle sequencing reactions used the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA) using the thermocycler parameters 94°C for 5 min., 50 cycles of 94°C for 1 min., and final elongation at 60°C for 10 min. Samples were electrophoresed on a Beckman Coulter CEQ 8000 sequencer Applied Biosystems 3730xl automated DNA sequencing instrument, using 96 cm capillary arrays and POP-7 polymer. Additional sequences where obtained from Genebank and previous studies (Hall et al. 2011).

Table 1. Taxonomic and fruit trait diversity of the tribe Brassiceae. Taxonomic information is from Warwick et al. (2001). Fruit trait information is from multiple sources referenced in Hall et al. (2011).

Genera	# of Recognized Species	Genera in Phylogeny	# Sp. in Phylogeny	Joint	Abscission	Dehiscence	Pericarp
Ammosperma	2	No	0	0	0	0	0
Boleum (Vella)	1	Yes	1	0	0	1	1
Brassica	39	Yes	4	1	0	0	0
Cakile	7	Yes	7+	1	1	1	1
Carrichtera	1	No	0	0	NA	0	0
Ceratocnemum	1	No	0	1	1	1	0
Chalcanthus	1	No	0	NA	NA	NA	NA
Coincya	6	Yes	3	1	0	0	1
Conringia	6	No	0	0	0	0	0
Cordylocarpus	1	Yes	1	1	1	1	1
Crambe	40	Yes	3	1	1	1	1
Crambella	1	Yes	1	1	1	1	1
Didesmus	2	Yes	2	1	1	1	1
Diplotaxis	32	Yes	2	1	0	0	1
Douepea	2	No	0	0	0	0	0
Enarthrocarpus	5	Yes	1	1	1	1	1
Eremophyton	1	No	0	1	NA	1	NA
Eruca	4	Yes	2	0	0	0	1
Erucaria	10	Yes	6	1	1	1	1
Erucastrum	25	Yes	6	0	0	0	1
Fezia	1	No	0	1	NA	NA	NA
Foleyola	1	No	0	1	NA	NA	NA
Fortuynia	2	No	0	1	1	1	1
Guiraoa	1	Yes	1	1	0	0	1
Hemicrambe	3	Yes	1	1	0	0	1
Henophyton	2	Yes	1	0	0	0	1
Hischfeldia	1	Yes	1	1	0	0	1
Krameriella	1	Yes	1	1	1	1	1
Moricandia	8	Yes	1	0	0	0	1
Morisia	1	Yes	1	1	1	0	1
Muricaria	1	Yes	1	1	1	1	1
Orychophragmus	2	No	0	0	0	0	0
Otocarpus	1	No	0	NA	NA	NA	NA

Genera	# of Recognized Species	Genera in Phylogeny	# Sp. in Phylogeny	Joint	Abscission	Dehiscence	Pericarp
Physorhynchus	2	No	0	1	NA	1	NA
Pseuderucaria	2	Yes	1	0	0	0	1
Pseudofortuynia	1	No	0	NA	NA	NA	NA
Psychine	1	Yes	1	0	0	0	1
Quezeliantha	1	No	0	NA	NA	NA	NA
Raffenaldia	2	Yes	1	0	0	1	1
Raphanus	3	Yes	1	1	1	1	1
Rapistrum	2	Yes	2	1	1	1	1
Rytidocarpus	1	Yes	1	0	0	0	1
Savignya	1	No	0	1	0	1	1
Schouwia	1	Yes	1	0	0	0	0
Sinapidendron	4	Yes	1	0	0	0	1
Sinapis	4	No	0	0	0	0	0
Succowia	1	No	0	1	1	1	1
Trachystoma	3	Yes	1	1	1	NA	0
Vella	7	Yes	1	0	0	0	1
Zilla	2	Yes	1	0	0	1	1

Data were analyzed using PE-Biosystems version 3.7 of the program Sequencing Analysis at the DNA Core facility of the University of Missouri, Columbia. DNA sequences were edited and aligned using SeqMan (DNAStar Inc., Madison Wisconsin) and aligned using MUSCLE (Edgar 2004). All sequences were submitted to NCBI—GenBank and gene alignments were submitted to TreeBASE (http://treebase.org).

Phylogenetic inference. The three intergenic regions of the chloroplast were concatenated as one data set, taking into account the uniparental inheritance and lack of recombination in the genome. For parsimony analysis, individual bases were considered multistate, unordered characters of equal weight; unknown nucleotides were treated as uncertainties. We inferred relationships from the nucleotide data using maximum likelihood (ML), and Bayesian inference (BI). Phylogenies were generated on the Cyberinfra- structure for Phylogenetic Research (CIPRES) portal 3 teragrid (http://www.phylo.org) (Miller et al. 2010). Phylogenetic analyses were rooted using 3 outgroups chosen based on recent phylogenies published for the family (Beilstein et al. 2008; Hall et al. 2011).

For both maximum likelihood (ML) and Bayesian methods (BI) the optimal model of sequence evolution was found for each marker using jModeltest (Posada 2008) and applied to each region independently within the concatenated sequence. Maximum likelihood and ML bootstrapping (MLB), with

different models allowed for each gene partition, was completed using GARLI 2.0 (Genetic Algorithm for Rapid Likelihood Inference) (Zwickl 2006). Bayesian analyses were conducted in BEAST v. 1.7.2 (Drummond and Rambaut 2007), allowing different models for each region and using default priors (Ronquist 2004; Alfaro and Holder 2006). Two independent runs of 5 X 10^7 generations were completed. Trees were sampled every 5,000 generations. The first 10% of runs were discarded as burn-in. The remaining trees from both runs were combined using LogCombiner v1.7.2. A majority-rule consensus tree with a 50% threshold based on posterior probabilities (PP) was constructed with TreeAnnotator v1.7.2.

Divergence time estimates. To estimate divergence times, we used a penalized maximum likelihood approach using r8s v. 1.71 (Sanderson 2003) and Bayesian approaches in BEAST v. 1.7.2 (Drummond and Rambaut 2007). These methods account for variation in substitution rates among branches on the tree. We calibrated the divergence time using a recently published fossil for the family in the genus *Thlaspi primaevum* (dated at 30.8--29.2 Mya) and secondary calibration points for the age of Lineage II (LINII) and the tribe Brassiceae (Beilstein et al. 2010). A detailed r8s analysis for the entire chloroplast dataset for the Brassiceae and twelve outgroups was conducted. We performed several analyses using r8s to explore the impact of placing the *Thlaspi primaevum* constraint (29.2 Mya) at

different nodes among outgroups. Additionally, we used two maximum age constraints (Brassiceae: 28.3 Mya; LINII: 37.8 Mya), two minimum age constrains (Brassiceae: 15.6 Mya; LINII: 23.7 Mya) or a combination of both, plus the fossil calibration.

We allowed BEAST to infer topology, branch lengths, and dates for the 3 chloroplast markers, 3 nuclear markers, and 6 combined markers. All our analyses in BEAST accounted for rate variation using an uncorrelated relaxed clock drawn from a lognormal distribution that allows different rates to be optimized independently on each branch of the tree. The Yule process described the likelihood of speciation, and branching rates were determined under substitution models determined by jModeltest (see above). In the analysis of combined data we unlinked the substitution model and kept the same clock model, allowing each data partition to evolve independently across the same tree. A normal distribution with lower and upper bounds was set for the age of the Lineage II (mean=43.2 mya, SD=0.5, bound=37.0 mya, upper bound=50.7 mya) and the age of the tribe Brassiceae (mean=30.8 Mya, SD=0.5, lower bound=23.7 mya, upper bound=37.8 mya). Lower and upper bounds were derived from 95% confidence intervals of the original estimates (Beilstein et al. 2010). The normal distribution is useful for imposing a prior with secondary calibration dates and minimum and maximum bounds (Ho and Phillips 2009).

Dating of the tree with BEAST was done simultaneously with the phylogenetic estimates described above. We ran two independent runs for 5 X 10^7 generations, saving data every 5,000 generations. We combined data from both runs using LogCombiner v. 1.7.2. We used TreeAnnotator v. 1.7.2 to produce maximum clade credibility trees from posterior probabilities and to determine the 95% probability density of ages for all nodes in the tree.

Fruit, Seed, Habitat, Range, and Climatic Data. All taxa were scored for four dispersal-related fruit characters: *joint* (present/absent), *joint abscission* (present/absent), *dehiscence* (fully or partially dehiscent vs. non-dehiscent), and *pericarp features* (present/absent; encompassing wings, hooks, and corkiness) (Table 1, Table 2). The majority of data on heteroarthrocarpy (dehiscence, joint, and abscission) were taken from Hall et al. (2011) or references therein. At the generic level, taxa used in this study were representative of the overall proportion of fruit trait diversity observed in this tribe for joint (represented 59% / overall 59%; Table 1), but slightly higher for joint abscission (41% / 39%; Table 1) and pericarp features (91% / 76%; Table 1), and lower for dehiscence (45% / 47%; Table 1). At the specific level, taxa used in this study were representative of the overall proportion of fruit trait diversity observed in this tribe for joint (67% / 69%; Table 1), but slightly higher or dehiscence (50% / 36%; Table 1) and pericarp features (90% / 76%; Table 1), and lower for joint abscission (47% / 68%; Table 1).

Seed mass and seed number data (Table 2) were collected from a combination of taxa from plants grown in the greenhouse, the Kew SEED Database, or the literature. For seed data measured on living specimens, data were collected across two periods. The majority of data collection occurred in 2004 with plants grown in the Harvard University Glasshouse. A subset of plants was subsequently grown in the Duke Greenhouses in 2010. In both cases, 1-6 individual plants were grown per taxon. Flowers were self- and cross-pollinated by hand to assure fruit set. 2-7 fruits were collected per individual and used to score fruit and seed traits. Seed number was counted per fruit and averaged across fruits and individuals for each taxon. Individual seed mass was measured as the mass (g) of a group of seeds (2-85) divided by the total number of seeds weighed. For a small number of taxa that we were not able to grow, we obtained additional measures of seed number from a collection for regional flora (see Hall et al. 2011 for references). Additionally, we collected seed number and seed mass data from the Kew Seed Information Database (http://data.kew.org/sid/).

Geo-coordinates for each taxon were obtained from the Global Biodiversity Information Society (http://www.gbif.org/), herbarium records, and personal observation. Geo-coordinates were used to calculate latitudinal range as the difference between the minimum and maximum latitude.

Table 2. Summary of fruit and seed traits used in analyzes. Fruit traits were scored as present (1) or absent (0). Seed traits represent means estimated from either direct measurement of fruit and seeds or taken from the literature. Source material include regional floras (Lit., see Chapter 2, Material and Methods), measurements taken by Jocelyn Hall in 2004 at Harvard University (JH), the Kew Gardens SEED Database (KEW), and measurements taken by Charles Willis in 2010 at Duke University (CW).

		Fruit Traits				*Seed Traits*					
Species Name	**Accession Name and Code**	**Joint**	**Abscission**	**Dehiscence**	**Pericarp**	**Seed Number (proximal segment)**	**Seed Number (distal segment)**	**Seed Number (total)**	**Seed Mass (g)**	**Seed Number (source)**	**Seed Mass (source)**
Boleum asperum Desv.	Boleum asperum 1587	0	0	1	1	2.0	0.0	2.0	NA	Lit.	NA
Brassica barrelieri (L.) Janka	Brassica barrellieri 1160	1	0	0	1	6.0	1.0	7.0	0.63	Lit.	CW
Brassica nigra (L.) Koch	Brassica nigra 0049	0	0	0	1	7.5	0.0	7.5	1.38	JH	JH
Brassica oleracea L. var. *acephala* DC.	Brassica oleracea 1284	1	0	0	1	19.0	1.0	20.0	4.04	Lit.	CW
Brassica spinescens Pomel.	Brassica spinscens 1800	1	0	0	1	19.9	1.0	20.9	0.41	JH	JH
Cakile arabica	Cakile arabica	2	1	1	1	0.7	1.0	1.7	1.89	JH	CW
Cakile artica Pobedimova	Cakile arctica	2	1	1	0	1.5	1.5	3.0	NA	Lit.	NA
Cakile constricta Rodman	Cakile constricta BS	2	1	1	0	1.0	1.3	2.2	3.23	JH	JH
Cakile edentula (Bigel.) Hook. ssp. *edentula*	Cakile edentula KIT	2	1	1	0	1.0	1.3	2.3	9.10	Lit.	Kew
Cakile lanceolata ssp. *fusiformis*	Cakile lanceolata GK	2	1	1	0	1.0	1.2	2.2	5.89	JH	JH
Cakile maritima Scopoli	Cakile maritima TAR	2	1	1	0	0.9	1.3	2.1	16.70	JH	Kew

Species Name	Accession Name and Code	Joint	Abscission	Dehiscence	Pericarp	Seed Number (proximal segment)	Seed Number (distal segment)	Seed Number (total)	Seed Mass (g)	Seed Number (source)	Seed Mass (source)
Coincya longirostra (Boiss.) Gr.&Burd.	Coincya longirostra 1175	1	0	0	1	6.0	6.0	12.0	0.62	Lit.	CW
Coincya monensis (L.) Gr.&Burd.	Coincya monensis 4429	1	0	0	1	18.6	1.8	20.4	0.97	JH	JH
Cordylocarpus muricatus Desf.	Cordylocarpus muricatus 1137	2	1	1	1	2.0	1.0	3.0	NA	Lit.	NA
Coincya rupestris Rouy	Coincya rupestris 1577	1	0	0	1	NA	NA	NA	NA	Lit.	NA
Crambe abyssinica Hochst.	Crambe abyssinica	2	1	1	1	0.0	NA	1.0	3.23	Lit.	CW
Crambe maritima L.	Crambe maritima 0510	2	1	1	0	0.0	NA	1.0	72.61	Lit.	Kew
Crambe orientalis L.	Crambe orientalis 3696	2	1	1	1	0.0	NA	1.0	3.43	Lit.	CW
Crambella teretifolia (Batt.) Maire	Crambella teretifolia 1971	2	1	1	1	0.0	NA	1.0	2.81	JH	JH
Didesmus aegyptius (L.) Desv.	Didesmus aegyptius 7320	2	1	1	1	0.6	0.8	1.4	0.21	Lit.	CW
Diplotaxis assurgens (Del.)Thell	Diplotaxis assurgens 1120	1	0	0	1	22.1	1.0	23.0	0.14	JH	JH
Didesmus bipinnatus (Desf.) DC.	Didesmus bipinnatus 1853	2	1	1	1	1.0	0.9	1.9	0.41	JH	JH
Diplotaxis harra ssp. *numidica* Mart. & Lab.	Diplotaxis harra 1831	0	0	0	1	70.3	0.0	70.3	0.10	JH	Kew
Enarthrocarpus lyratus (Forsk.) DC.	Enarthrocaprus lyratus 1206	2	1	1	1	1.9	3.5	5.4	1.12	JH	JH
Erucastrum abyssinicum (A. Rich) O.E. Shulz	Erucastrum abyssinicum 0430	0	0	0	1	1.8	0.0	1.8	0.46	Lit.	CW
Erucaria cakiloidea (DC.) O.E.Schylz	Erucaria cakiloidea 3738	2	1	1	1	3.2	2.5	5.7	0.69	JH	JH
Erucastrum canariense Webb & Berth.	Erucastrum canariense 5305	0	0	0	1	16.6	0.0	16.6	0.14	Lit.	CW
Erucastrum cardaminoides (Weed) O.E.Schulz	Erucastrum cardaminoides 1070	0	0	0	1	9.0	0.0	9.0	0.26	JH	JH

Species Name	Accession Name and Code	Joint	Abscission	Dehiscence	Pericarp	Seed Number (proximal segment)	Seed Number (distal segment)	Seed Number (total)	Seed Mass (g)	Seed Number (source)	Seed Mass (source)
Erucastrum elatum (Ball) O.E. Schulz [laevigatum Marie & Weiller]	Erucastrum elatum 4127	1	0	0	1	15.4	0.9	16.3	0.36	JH	JH
Erucaria erucarioides (Coss.&Dur.) Mueller	Erucaria erucarioides 1944	1	0	0	1	9.5	1.2	10.7	0.36	JH	JH
Erucastrum gallicum (Willd.) O.E. Schulz	Erucastrum gallicum 1209	0	0	0	1	25.0	0.0	25.0	0.08	Lit.	CW
Erucaria hispanica (L.) Druce	Erucaria hispanica 2055	1	0	0	1	4.0	1.3	5.3	0.30	JH	Kew
Erucaria microcarpa Boiss.	Erucaria microcarpa 4620	2	1	0	1	4.0	0.5	4.5	0.14	Lit.	CW
Erucaria ollivieri Maire	Erucaria ollivieri 2983	2	1	0	1	0.9	1.6	2.5	0.41	JH	JH
Eruca pinnatifida f . Aurea (Batt.) Maire	Eruca pinnatifida 1471	0	0	0	1	34.8	0.0	34.8	0.35	Lit.	JH
Erucaria pinnata (Viv.) Tackh.&Boulos	Erucaria pinnata 1851	2	1	1	1	1.6	2.4	4.0	1.72	JH	JH
Eruca vesicaria [sativa]	Eruca vesicaria 3750	0	0	0	1	17.6	0.0	17.6	0.60	JH	Kew
Erucastrum virgatum ssp *baeticum* (Boiss.) Gomez-Campo	Erucastrum virgatum 5364	1	0	0	1	5.5	0.5	6.0	0.19	JH	JH
Guiraoa arvensis Cosson	Guiraoa arvensis 1550	1	0	0	1	1.7	1.6	3.3	0.43	JH	JH
Hemicrambe fruticulosa Webb	Hemicrambe fruticulosa 2232	1	0	0	1	0.5	4.0	4.5	1.49	Lit.	CW
Henophyton deserti (Coss. & Dur.) Coss. & Dur.	Henophyton deserti 1945	0	0	0	1	21.0	0.0	21.0	NA	Lit.	NA
Hirschfeldia incana (L.) Lagreze-Fossat	Hirschfeldia incana 2024	1	0	0	1	7.5	0.9	8.3	0.40	JH	Kew
Kremeriella cordylocarpus (Coss.&Dur.) Maire	Kremeriella cordylocarpus 1142	2	1	1	1	0.0	NA	1.0	NA	Lit.	NA

Species Name	Accession Name and Code	Joint	Abscission	Dehiscence	Pericarp	Seed Number (proximal segment)	Seed Number (distal segment)	Seed Number (total)	Seed Mass (g)	Seed Number (source)	Seed Mass (source)
Moricandia arvensis (L.) DC.	Moricandia arvensis 0863	0	0	0	1	64.7	0.0	64.7	0.25	Lit.	CW
Morisia monanthos (Viv.) Asch.	Morisia monanthos 3816	2	1	0	1	7.0	2.0	9.0	0.81	Lit.	CW
Muricaria prostrata (Desf.)Desv.	Muricaria prostrata 1855	2	1	1	1	0.0	NA	1.0	0.16	Lit.	CW
Psychine stylosa Desf.	Psychine stylosa 1458	0	0	0	1	23.0	0.0	23.0	0.57	JH	JH
Pseuderucaria teretifolia (Desf.) Pomel	Pseuderucaria teretifolia 1844	0	0	0	1	55.8	0.0	55.8	0.21	JH	JH
Rapistrum perenne (L.) All.	Rapistrum perenne 1404	2	1	1	1	1.0	1.0	2.0	NA	Lit.	NA
Raffenaldia primuloides Godr.	Raffenaldia primuloides 4386	0	0	1	1	6.0	0.0	6.0	NA	Lit.	NA
Raphanus raphanistrum L.	Raphanus raphanistrum 1509	2	1	1	1	0.3	4.3	4.5	21.58	Lit.	Kew
Rapistrum rugosum (L.) All.	Rapistrum rugosum 1527	2	1	0	1	1.0	0.9	1.9	0.98	JH	JH
Rytidocarpus moricandioides Cosson	Rytidocarpus moricandioides 0708	0	0	0	1	26.5	0.0	26.5	0.51	JH	JH
Schouwia thebaica Webb.	Schouwia thebaica 5780	0	0	0	0	40.9	0.0	40.9	3.60	Lit.	CW
Sisymbrium altissimum L.	Sisymbrium altissimum 1724	0	0	0	1	78.1	0.0	78.1	0.20	Lit.	Kew
Sinapidendron angustifolium (DC.) Lowe	Sinapidendron angustifolium 3620	0	0	0	1	27.6	0.0	27.6	0.25	JH	JH
Stanleya pinnata (Pursh) Britton	Stanleya pinnata 1735	0	0	0	1	110.0	0.0	110.0	1.33	Lit.	Kew
Trachystoma labassii Maire	Trachystoma labassii 3014	1	1	NA	0	2.0	10.0	12.0	NA	Lit.	NA
Vella spinosa Boiss.	Vella spinosa 2007	0	0	0	1	2.0	0.0	2.0	1.80	Lit.	CW
Zilla spinosa (L.) Prantl.	Zilla spinosa 0731	0	0	1	1	2.0	0.0	2.0	1.30	Lit.	CW

Climatic data were collected for each taxon from the WorldClim Global Climate Database (http://www.worldclim.org/). These data include 19 climatic variables as well as altitude. We used Maxent 3.3.2 (Phillips and Dudik 2008) to extract all 19 climate plus altitude variables from the BioClim data file based on geo-coordinates at a resolution of ~ 1 km^2. The median of these observations was taken per taxon per variable. To reduce the effect of auto-correlation between climate variables, we performed principal component analysis on the median values of all 19 climate variables and altitude across all taxa using the 'principal' function with 'varimax' rotation in R v. 2.13 (R Core Team 2013) (Table 3).

Taxa were also scored for habitat. Habitats included 'field', 'ruderal', 'desert', and 'coastal'. Taxa were scored for each of these habitats based on descriptions from regional floras (see Hall et al. 2011).

Fruit Trait, Seed, Habitat, Range, and Climate Correlations. To test for correlated evolution among dispersal-related fruit characters and between fruit traits and habitat, we used Pagel's (1994) method for testing for correlated evolution of binary traits. This method compares two evolutionary models: one in which the evolution of the two traits is independent and one in which transitions between states in one trait can depend on transitions between states in the other trait. If the dependent model is more likely, then the traits are considered to be correlated. Maximum likelihood was used to estimate model

parameters. A log-likelihood ratio test was used to test for significant differences between the models.

To test for the correlated evolution of dispersal-related fruit characters with seed and environmental traits, we used phylogenetic generalized least squares (PGLS) models implemented in the R package 'caper' v0.5 (Orme et al. 2012). PGLS models are equivalent to general linearized models with the addition that they account for phylogenetic covariance among taxa. However, controlling for phylogenetic co-variance when analyzing a trait with little or no phylogenetic signal can lead to errors in the estimation of regression parameters (Revell 2010). PGLS models, as implemented in 'caper', simultaneously estimate phylogenetic signal (λ) and regression parameters, and corrects for phylogeny covariance only as much as the estimation of λ suggests, thereby reducing the error resulting from over-correction. If, for example, λ is effectively 0, then the PGLS model will behave as a traditional general linearized model. PGLS models were run with seed traits and environmental data as the dependent variable and the fruit character as the independent variable. To correct for multiple comparisons across several geo-spatial and environmental independent variables, we corrected significance levels by the number of comparisons using a Bonferonni correction.

Table 3. Principle component loadings and proportion variance for climate and altitude data for the Brassiceae.

Variables	PC1	PC2	PC3	PC4	PC5
Altitude				0.99	
Mean annual precipitation		0.93			
Diurnal range	0.51			0.62	
Isothermality			0.61		
Max. temp. wettest month	0.87				
Mean annual temperature	0.88				
Mean temp. coldest quarter	0.72		0.65		
Mean temp. driest quarter	0.88				
Mean temp. wettest quarter	0.95				
Min. temp. coldest month	0.62		0.76		
Percip. coldest quarter					0.83
Percip. driest month		0.56			
Percip. driest quarter		0.57			
Seasonal percip. variation	0.55				
Percip. wettest month		0.91			
Percip. wettest quarter		0.83			
Percip. warmest quarter		0.93			
Annual temp. range			-0.88		
Seasonal temp. variation			-0.93		
Proportion variation	*0.90*	*0.05*	*0.02*	*0.01*	*0.01*

Trait-Dependent Diversification Rates. To test if diversification rates were associated with the evolution of dispersal-related fruit traits and habitat shifts, we first compared diversification rates between character states for all joint, indehiscence, pericarp features, using both the binary state speciation and extinction model (BiSSE) and multiple state speciation and extinction model (MuSSE) (Maddison et al. 2007), implemented in the R package 'diversitree' v. 0.9-3 (FitzJohn 2012). BiSSE and MuSSE estimate and compare speciation and extinction rates among character states. In both cases, we used both ML and MCMC methods to estimate diversification parameters. We tested if rates of speciation and extinction were significantly different between characters states by using a log-likelihood test of models when either lambda or mu was constrained, versus when they were allowed to vary. For diversification analyzes, we corrected for incomplete sampling by specifying the fraction of unsampled species that had each given character state (FitzJohn 2012). To compare diversification across habitat types, however, we accounted only for the overall fraction of unsampled species, given the challenges of identifying habitat types for rare or poorly study taxa. For these analyses, we accounted for phylogenetic uncertainty as explained next.

Phylogenetic uncertainty. To address the influence of branch length and topological uncertainty on our both correlated and diversification results, we ran

every analysis across a subset of 100 ML and Bayesian divergence-time estimated trees sampled from bootstrap and posterior distributions, respectively. Median values for every estimate were computed across all 100 analyzes. Median results did not differ qualitative in terms of significance or direction between ML and Bayesian tree sets, thus only median values from the Bayesian tree set are presented.

2.3 Results

Phylogeny. Combined marker analysis resulted in the greatest resolution across the tribe for both Bayesian and maximum-likelihood analysis (Figures 1 and 2). In contrast to Hall et al. (2011), Bayesian analysis resolved the genus *Cakile* as monophyletic, with *Cakile arabica* appearing as the basal lineage (Figure 1). This result, however, was not supported by ML analysis (Figure 2). Separate analysis of nuclear and chloroplast markers resulted in largely concordant trees (Figures 3, 4, 5 and 6). Similarly, our tree topologies were largely concordant with those reported by Hall et al. (2011). Nuclear markers gave greater resolution at more recent nodes, especially within the subtribe Cakilinae (Figures 3 and 4), while chloroplast markers resulted in greater resolution of deeper nodes (Figures 5 and 6).

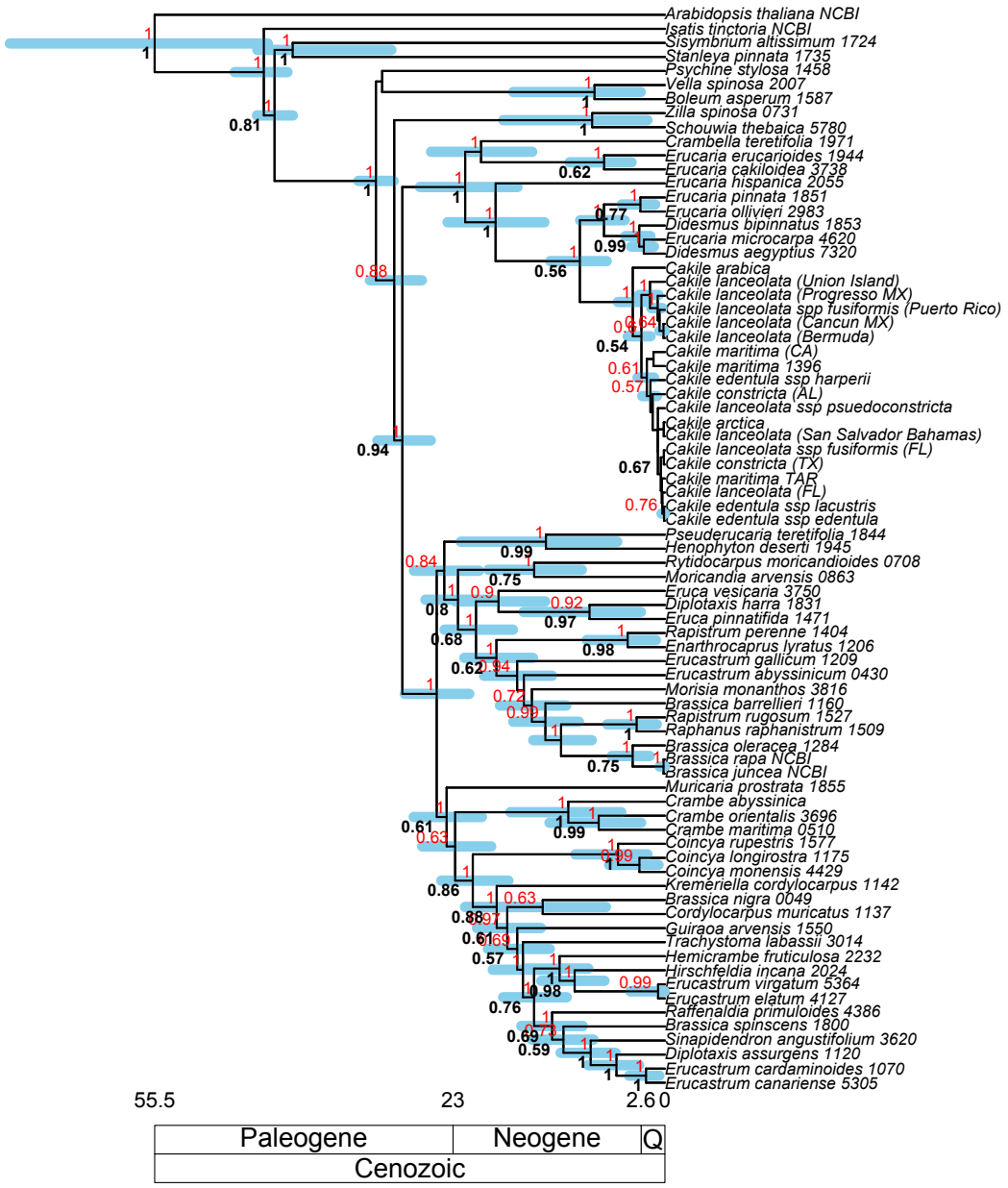


Figure 1. Bayesian estimated phylogeny of Brassiceae based on *matK*, *psbA*, *trnG* (chloroplast) and *ITS*, *Fnr*, *phyA* (nuclear) markers. Posterior probabilities > 0.50 are indicated above branches in red. Maximum-likelihood boost-strap values for nodes with > 0.50 support are in black. Branch lengths are proportional to time based on divergence time estimates and scale to millions of years. Blue bars indicate 95% confidence interval of divergence time estimate. Time scale in millions of years with geological periods is below tree.

Figure 2. Maximum-likelihood estimated phylogeny of Brassiceae based on *matK*, *psbA*, *trnG* (chloroplast) and *ITS*, *Fnr*, *phyA* (nuclear) markers. Posterior probabilities > 0.50 are indicated above branches in red. Maximum-likelihood boost-strap values for nodes with > 0.50 support are in black.

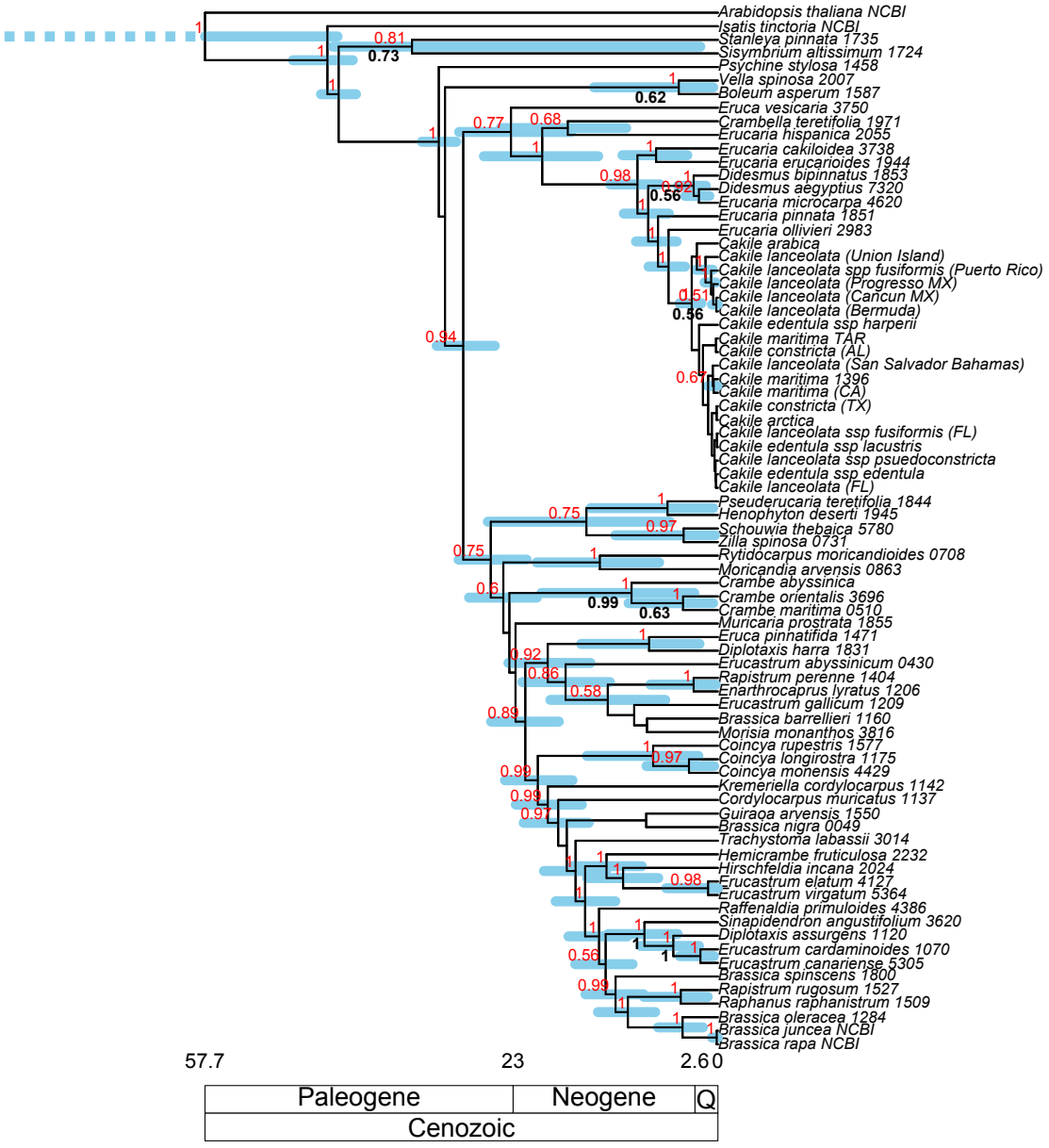


Figure 3. Bayesian estimated phylogeny of Brassiceae based on *ITS, Fnr, phyA* (nuclear) markers. Posterior probabilities > 0.50 are indicated above branches in red. Maximum-likelihood boost-strap values for nodes with > 0.50 support are in black. Branch lengths are proportional to time based on divergence time estimates and scale to millions of years. Blue bars indicate 95% confidence interval of divergence time estimate. Dotted blue line indicates that the bound of the confidence interval is beyond the display of the figure. Time scale in millions of years with geological periods is below tree.

Figure 4. Maximum-likelihood estimated phylogeny of Brassiceae based on *ITS, Fnr, phyA* (nuclear) markers. Posterior probabilities > 0.50 are indicated above branches in red. Maximum-likelihood boost-strap values for nodes with > 0.50 support are in black.

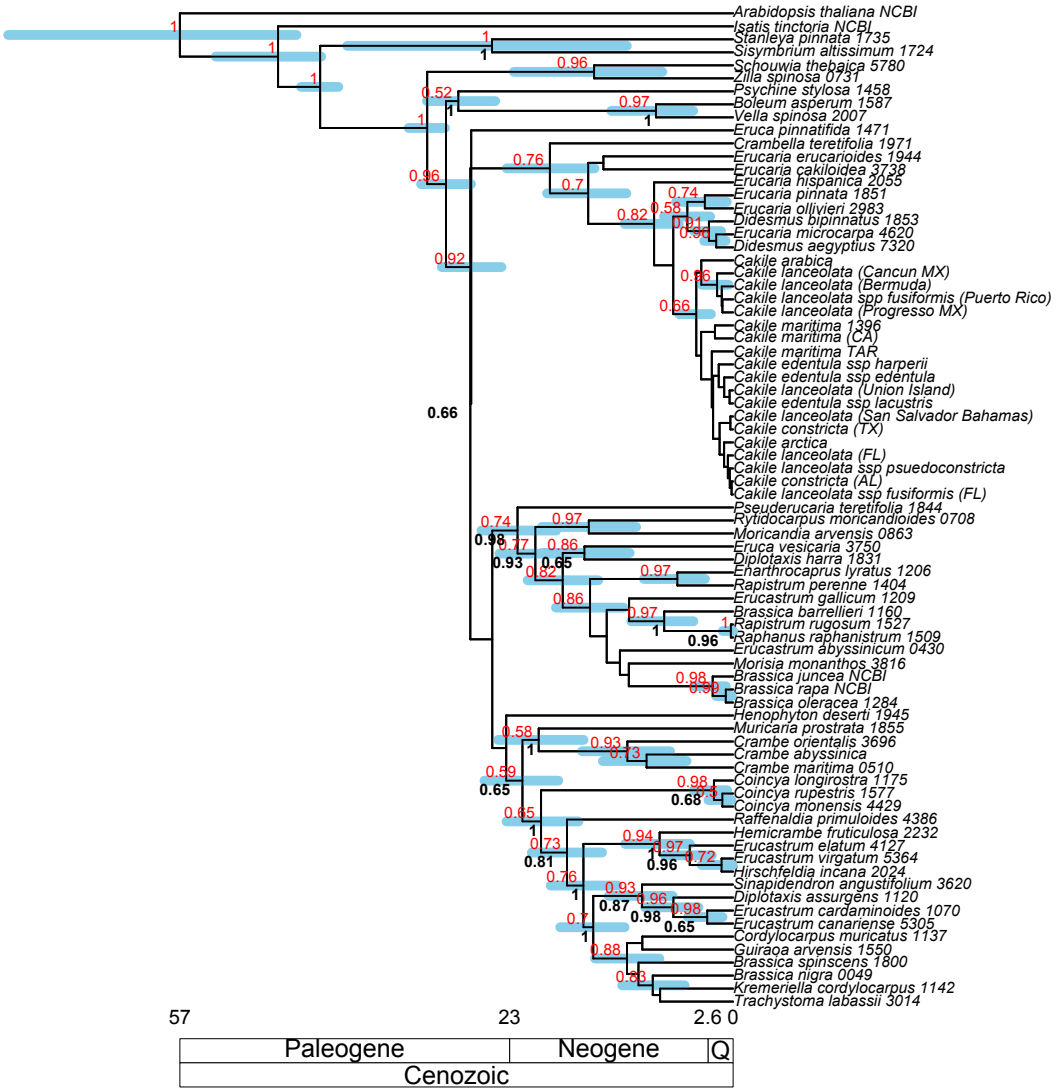


Figure 5. Bayesian estimated phylogeny of Brassiceae based on *matK, psbA, trnG* (chloroplast) markers. Posterior probabilities > 0.50 are indicated above branches in red. Maximum-likelihood boost-strap values for nodes with > 0.50 support are in black. Branch lengths are proportional to time based on divergence time estimates and scale to millions of years. Blue bars indicate 95% confidence interval of divergence time estimate. Time scale in millions of years with geological periods is below tree.

Figure 6. Maximum-likelihood estimated phylogeny of Brassiceae based on *matK*, *psbA*, *trnG* (chloroplast) markers. Posterior probabilities > 0.50 are indicated above branches in red. Maximum-likelihood boost-strap values for nodes with > 0.50 support are in black.

Evolution of Fruit and Seed Traits. Fruit traits were significantly associated with one another (Table 4). The evolution of a joint was significantly associated with the evolution of an abscission zone (Table 4). The evolution of indehiscence was also associated with the evolution pericarp features (Table 4), which is to be expected, since pericarp features seem unlikely to evolve if they do not surround the dispersing propagule. More interestingly, the evolution of indehiscence was significantly associated with the evolution of an abscission zone on the joint, which was marginally significantly associated pericarp features (Table 4), suggesting that when indehiscence evolves to enclose proximal segments, other features evolve that enhance dispersal. In contrast, there was no association between the evolution of a joint *per se* (including non-abscising joints) and indehiscence or pericarp features, suggesting that heteroarthrocarpy itself (defined by the joint) does not necessarily alter dispersal via other traits. Dispersal-related modifications of heteroarthrocarpy however, such as indehiscence or joint abscission, appear to have promoted the evolution of the tightly linked complex of indehiscence, abscission and pericarp features.

Seed size was significantly negatively correlated with seed number per fruit (PGLM: $\beta = -0.48 \pm 0.17$, $p < 0.01$). Consistent with this association, fruit traits that were positively associated with seed size were negatively associated with seed number per fruit (Table 5). The evolution of a joint *per se* was not associated

with seed size or number (Table 5). However, the subsequent evolution of an abscission zone, indehiscence, and pericarp features were associated with an increase in seed size and a decrease in seed number, although in the case of pericarp features the decrease in seed number was not significant (Table 5). Total biomass per fruit was not associated with fruit traits (Table 5). As above, the evolution of heteroarthrocarpy *per se* (defined by the presence of a joint), does not appear to have altered the evolution of seed size or number, but seed size and number do seem to have coevolved with traits associated with dispersal that follow the evolution of heteroarthrocarpy (Table 5).

Evolution of Fruit and Seed Traits with Habitat, Climatic Niche and Range. Habitat was not associated with the evolution of any fruit trait (Table 6). However, seed traits were associated with habitat (Table 7). Coastal taxa had marginally significantly larger seeds than those found in fields, deserts and ruderal habitats (Table 7). Desert and coastal taxa also had significantly or marginally significantly more seeds per fruit than taxa that occur in fields (Table 7). For coastal taxa, however, the pattern of seed number was possibly driven by a single taxon, *Brassica oleracea*, with a large number of seeds per fruit, and its exclusion as coastal species makes the difference non-significant. Total seed biomass per fruit was maintained across all habitats except the desert, where there was a marginally significant reduction (Table 7).

Table 4. Test of correlated evolution of fruit traits and habitat. The test consists of comparing likelihoods of two models: one in which transitions between two traits are dependent on one another, and the other in which the transitions are independent of one another. A significant difference between models indicates that the traits are evolutionarily linked (significant results in bold). Relative transition rates between trait pairs are on the left. Significance was adjusted for multiple comparisons (3 per trait) using Bonferonni correction ($\alpha_{0.05}$ = 0.0167). Significant associations are in bold (P < 0.0167, corresponding to unadjusted significance of P < 0.05), and marginally significant associations are in italics (P< 0.033, corresponding to unadjusted significance of P < 0.1). Values represent the median estimate from analysis conducted across 100 trees sampled from the Bayesian posterior distribution.

Trait A	Trait B	Log Likelihood		ΔlnLik	P	Dependent Transition Rates							
		Dependent	Independent			0,0 -> 0,1	0,0 -> 1,0	0,1 -> 0,0	0,1 -> 1,1	1,0 -> 0,0	1,0 -> 1,1	1,1 -> 0,1	1,1 -> 1,0
Abscission Zone	Pericarp Features	-74.36	-78.66	*-4.29*	*0.0293*	12.8	29.3	71.2	68.4	70.0	57.7	34.8	56.7
Abscission Zone	Indehiscence	-67.11	-80.85	**-13.74**	**0.0000**	9.8	11.8	71.4	71.1	70.5	69.4	15.3	18.9
Indehiscence	Pericarp Features	-69.13	-77.24	**-8.10**	**0.0012**	9.1	20.7	71.3	67.8	68.3	67.2	23.5	47.3
Joint	Abscission Zone	-68.40	-80.50	**-12.10**	**0.0000**	0.0	40.2	0.0	0.0	50.2	41.0	0.0	26.1
Joint	Pericarp Features	-75.80	-76.39	-0.59	0.1641	15.2	65.4	71.2	71.9	50.4	43.1	18.4	67.7
Joint	Indehiscence	-77.02	-79.42	-2.39	0.1093	15.8	57.4	70.9	72.2	53.8	60.0	14.0	58.2

Table 5. Evolution of Fruit and Seed Traits. Correlations were tested for using phylogenetic generalized linear models (PGLM). Fruit-Seed trait correlations were tested for using univariate and multivariate models. Significance was adjusted for multiple comparisons (3 traits) using Bonferonni correction ($\alpha_{0.05}$ = 0.0167). Significant associations are in bold (P < 0.0167, corresponding to unadjusted significance of P < 0.05), and marginally significant associations are in italics (P < 0.033, corresponding to unadjusted significance of P < 0.1). Seed mass and total seed biomass are log-transformed (g). Values represent the median estimate from analysis across 100 trees sampled from the Bayesian posterior distribution.

	Seed Mass				Total Seed Number per Fruit				Total Seed Biomass per Fruit			
	β	SE	t	P	β	SE	t	P	β	SE	t	P
(Intercept)	-0.71	0.65	-1.09	0.2803	3.12	0.59	5.33	0.0000	1.98	0.34	5.81	0.0000
joint, no abscission	0.44	0.49	0.89	0.3784	-0.41	0.35	-1.17	0.2489	-0.34	0.52	-0.65	0.5162
joint, abscission	1.48	0.54	**2.77**	**0.0080**	-1.65	0.41	**-4.01**	**0.0002**	-0.56	0.46	-1.21	0.2323
(Intercept)	-0.71	0.68	-1.04	0.3042	2.99	0.61	4.94	0.0000	2.15	0.61	3.52	0.0010
indehiscent	1.55	0.39	**4.00**	**0.0002**	-0.90	0.30	**-3.04**	**0.0038**	0.20	0.47	0.44	0.6653
(Intercept)	-0.61	0.66	-0.93	0.3582	2.88	0.69	4.15	0.0001	2.14	0.66	3.23	0.0022
pericarp features	1.49	0.37	**4.03**	**0.0002**	-0.36	0.27	-1.32	0.1943	0.82	0.45	1.82	0.0755
(Intercept)	-0.89	0.67	-1.33	0.1901	3.17	0.56	5.63	0.0000	2.24	0.73	3.09	0.0034
joint, no abscission	0.50	0.43	1.15	0.2559	-0.44	0.35	-1.27	0.2124	-0.01	0.53	-0.02	0.9844
joint, abscission	0.13	0.59	0.23	0.8211	-1.35	0.48	**-2.83**	**0.0069**	-1.12	0.73	-1.54	0.1316
indehiscent	1.21	0.47	*2.59*	*0.0130*	-0.40	0.36	-1.10	0.2759	0.60	0.64	0.95	0.3466
pericarp features	1.06	0.36	**2.91**	**0.0056**	-0.12	0.28	-0.43	0.6657	0.92	0.49	1.87	0.0685

There were no significant associations between fruit and seed traits and measured climate or altitude variables (Table 8). There was, however, a marginally significant positive association between seed size and latitudinal range (Table 8). Larger seeds tended to have wider ranges, suggesting the importance of establishment to range expansion after long-distance dispersal. This relationship held even when other fruit traits and seed number were included in the model, but it was not significant.

Trait- and Habitat-Dependent Diversification Rates. The evolution of fruit traits and the transition to coastal habitat were significantly associated with diversification rates (Table 9, Figure 7). The diversification rate of taxa that have a joint was higher than taxa without a joint (Table 9). This pattern, however, appears to be driven the evolution of an abscission zone. Taxa with an abscission zone was significantly greater than that of taxa with no joint or taxa with a joint, but no abscission zone (Table 9, Figure 7). The evolution of indehiscence and dispersal-related pericarp features were also associated with significantly greater diversification rates (Table 9, Figure 7). Although extinction rates did differ significantly between taxa with different habitat and joint states, differences in extinction rates were relatively minor compared to the magnitude of difference in speciation rates (Table 9). In all cases, increased speciation rates were responsible for the increased diversification rates illustrated in Figure 7.

Table 6. Test of correlated evolution of fruit traits and habitat. Test consists of comparing likelihoods of two models: one was transitions between two traits are dependent on one another, and a model where the transitions are independent of one another. A significant difference indicates that the traits are evolutionarily linked (sig. results in bold). Transition rates between trait-pairs can be found on the right. Significance adjusted for multiple comparisons (4 per trait) using Bonferonni correction ($\alpha_{0.05} = 0.0125$). Values represent the median estimate from analysis across 100 trees sampled from the Bayesian posterior distribution.

Trait A	Trait B	Log Likelihood		ΔlnLik	P	Dependent Transition Rates							
		Dependent	Independent			0,0 -> 0,1	0,0 -> 1,0	0,1 -> 0,0	0,1 -> 1,1	1,0 -> 0,0	1,0 -> 1,1	1,1 -> 0,1	1,1 -> 1,0
Abscission Zone	Coast	-61.39	-63.66	4.55	0.1170	4.80	38.72	72.73	68.74	70.13	21.64	28.36	67.78
Pericarp Features	Coast	-55.04	-58.65	7.22	0.0489	4.00	19.47	72.95	66.72	69.20	33.39	29.56	65.21
Indehiscence	Coast	-60.68	-61.77	2.18	0.1833	4.74	33.06	75.15	66.62	67.85	21.95	32.36	65.09
Joint	Coast	-63.79	-61.13	5.33	0.0927	4.79	70.34	70.55	70.19	46.76	15.46	14.68	68.06
Abscission Zone	Desert	-75.02	-74.02	2.00	0.1839	25.72	51.24	65.83	46.32	67.92	24.50	65.87	68.35
Pericarp Features	Desert	-71.49	-70.32	2.34	0.1815	30.54	34.38	68.44	22.53	67.30	19.50	69.73	70.35
Indehiscence	Desert	-73.28	-72.93	0.70	0.1234	33.07	54.64	65.20	29.91	68.22	16.04	71.76	66.93
Joint	Desert	-77.02	-72.93	8.19	0.0341	15.83	57.43	70.88	72.23	53.81	59.96	14.01	58.15
Abscission Zone	Field	-65.53	-66.38	1.69	0.1815	6.43	38.37	73.14	68.01	70.47	27.85	36.78	66.92
Pericarp Features	Field	-63.88	-64.42	1.10	0.1584	16.32	35.45	66.07	19.94	70.73	9.16	70.38	71.07
Indehiscence	Field	-64.93	-65.44	1.02	0.1528	8.04	37.78	71.23	63.27	72.96	23.61	43.74	66.64
Joint	Field	-65.11	-69.35	8.48	0.0305	7.77	68.17	71.72	70.62	48.03	19.00	21.74	67.55
Abscission Zone	Ruderal	-76.90	-79.98	6.15	0.0710	65.85	65.81	43.40	20.58	47.28	22.87	72.47	69.73
Pericarp Features	Ruderal	-76.85	-75.81	2.10	0.1837	56.57	30.45	65.67	26.46	65.35	50.59	67.07	65.14
Indehiscence	Ruderal	-77.79	-78.83	2.09	0.1838	64.82	59.68	53.17	27.06	61.28	32.62	69.45	69.10
Joint	Ruderal	-76.95	-78.50	3.09	0.1648	67.70	70.77	43.16	57.16	22.03	35.90	61.75	69.13

Table 7. Correlated Evolution of Habitat and Seed Traits. Correlations tested for using phylogenetic generalized linear models (PGLM). Significance adjusted for multiple comparisons (3) using Bonferonni correction ($\alpha_{0.05} = 0.0167$). Significant associations are in bold ($P < 0.0167$), and marginally significant associations are in italics ($P < 0.025$). Seed mass and total seed biomass were log-transformed (g). Values represent the median estimate from analysis across 100 trees sampled from the Bayesian posterior distribution.

	Seed Mass				Total Seed Number per Fruit				Total Seed Biomass per Fruit			
	β	**SE**	**t**	**P**	**β**	**SE**	**t**	**P**	**β**	**SE**	**t**	**P**
Field (Intercept)	0.31	0.44	0.70	0.4873	1.99	0.79	2.52	0.0163	0.39	1.13	0.35	0.7299
Ruderal	-1.08	0.52	-2.08	0.0444	0.86	0.43	1.98	0.0550	-2.37	1.33	-1.78	0.0833
Desert	-1.15	0.54	-2.14	0.0389	**1.06**	**0.35**	**2.99**	**0.0050**	*-3.42*	*1.37*	*-2.50*	*0.0173*
Coast	*1.56*	*0.66*	*2.38*	*0.0228*	*1.21*	*0.53*	*2.30*	*0.0274*	1.67	1.68	1.00	0.3260

Table 8. Correlated evolution of fruit traits, range and climate variables. Correlations were tested for using PGLM. Climate variables were reduced to five principle components. The major character of each component is listed in parentheses. Fruit-climate correlations were tested for using univariate and multivariate models. Significance adjusted for multiple comparisons (6 traits) using Bonferonni correction (α = 0.0083). Significant associations are in bold (P < 0.0083), and marginally significant associations are in italics (P < 0.0167). Values represent the median estimate from analysis across 100 trees sampled from the Bayesian posterior distribution.

	Latitudinal Range				PC1 (temperature)				PC2 (percipitation)				PC3 (seasonality)				PC4 (altitude)				PC5 (winter percipitation)			
	β	SE	t	P	β	SE	t	P	β	SE	t	P	β	SE	t	P	β	SE	t	P	β	SE	t	P
(Intercept)	2.34	0.59	3.98	0.0002	-0.07	0.24	-0.28	0.7774	-0.27	0.33	-0.82	0.4167	0.11	0.24	0.44	0.6600	0.26	0.20	1.32	0.1933	0.00	0.24	0.01	0.9948
joint, no abscission	-0.13	0.47	-0.27	0.7911	0.17	0.38	0.45	0.6554	0.54	0.37	1.44	0.1573	0.04	0.38	0.11	0.9127	-0.25	0.32	-0.77	0.4445	0.26	0.38	0.70	0.4902
joint, abscission	-0.38	0.49	-0.77	0.4442	0.00	0.32	-0.01	0.9938	0.38	0.34	1.10	0.2770	-0.17	0.32	-0.54	0.5920	-0.63	0.27	-2.35	0.0228	-0.15	0.32	-0.46	0.6484
(Intercept)	2.16	0.56	3.85	0.0003	-0.03	0.18	-0.15	0.8852	0.00	0.37	-0.01	0.9916	0.18	0.18	0.98	0.3335	0.13	0.16	0.81	0.4217	0.07	0.18	0.36	0.7202
indehiscent	0.22	0.38	0.57	0.5719	-0.01	0.28	-0.04	0.9718	-0.23	0.30	-0.77	0.4460	-0.33	0.28	-1.17	0.2456	-0.46	0.24	-1.89	0.0642	-0.17	0.29	-0.58	0.5624
(Intercept)	2.22	0.55	4.06	0.0002	-0.08	0.17	-0.48	0.6325	-0.17	0.30	-0.56	0.5789	0.02	0.17	0.12	0.9011	-0.01	0.15	-0.08	0.9346	0.09	0.17	0.54	0.5890
pericarp features	-0.02	0.38	-0.06	0.9521	0.17	0.31	0.55	0.5868	0.44	0.30	1.47	0.1481	0.07	0.31	0.22	0.8280	-0.17	0.27	-0.62	0.5378	-0.31	0.31	-1.02	0.3100
(Intercept)	2.29	0.66	3.49	0.0010	-0.10	0.25	-0.40	0.6891	-0.36	0.24	-1.55	0.1272	0.15	0.25	0.59	0.5572	0.28	0.21	1.34	0.1852	0.01	0.25	0.04	0.9681
joint, no abscission	-0.01	0.48	-0.01	0.9889	0.17	0.40	0.43	0.6714	0.37	0.37	1.01	0.3167	-0.06	0.39	-0.16	0.8734	-0.29	0.33	-0.87	0.3864	0.32	0.39	0.80	0.4257
joint, abscission	-0.95	0.61	-1.56	0.1251	-0.07	0.44	-0.16	0.8736	0.96	0.41	2.35	0.0227	0.14	0.43	0.31	0.7572	-0.50	0.37	-1.35	0.1828	-0.23	0.43	-0.54	0.5918
indehiscent	0.85	0.54	1.58	0.1205	0.00	0.49	-0.01	0.9940	-0.83	0.46	-1.81	0.0759	-0.65	0.48	-1.33	0.1888	-0.27	0.41	-0.65	0.5183	0.30	0.48	0.62	0.5366
pericarp features	-0.22	0.41	-0.54	0.5911	0.22	0.37	0.58	0.5618	0.78	0.35	2.22	0.0309	0.38	0.37	1.02	0.3106	0.13	0.31	0.42	0.6785	-0.36	0.37	-0.96	0.3431
(Intercept)	1.94	0.22	8.74	0.0000	-0.23	0.37	-0.62	0.5372	0.16	0.18	0.89	0.3803	-0.03	0.17	-0.16	0.8763	-0.19	0.13	-1.53	0.1340	-0.03	0.18	-0.15	0.8834
seed mass (g)	0.28	0.16	1.79	0.0815	-0.07	0.11	-0.64	0.5278	0.28	0.12	2.22	0.0324	-0.10	0.12	-0.83	0.4119	-0.06	0.09	-0.67	0.5071	-0.02	0.13	-0.12	0.9044
(Intercept)	1.63	0.40	4.09	0.0002	-0.26	0.50	-0.53	0.5985	0.52	0.31	1.67	0.1029	-0.04	0.29	-0.13	0.9010	-0.33	0.22	-1.55	0.1291	-0.03	0.32	-0.08	0.9370
seed number	0.12	0.17	0.70	0.4889	0.02	0.13	0.16	0.8776	-0.23	0.13	-1.74	0.0897	0.02	0.12	0.17	0.8694	0.08	0.09	0.90	0.3726	0.00	0.13	0.01	0.9913
(Intercept)	1.36	0.39	3.4857	0.0013	-0.17	0.49	-0.34	0.7383	0.38	0.32	1.19	0.24090	0.04	0.31	0.12	0.9076	-0.32	0.23	-1.39	0.1727	-0.01	0.34	-0.04	0.9691
seed mass (g)	*0.43*	*0.17*	*2.4705*	*0.0164*	-0.08	0.12	-0.64	0.5244	0.22	0.14	1.57	0.12540	-0.11	0.14	-0.84	0.4070	-0.03	0.10	-0.28	0.7787	-0.02	0.15	-0.13	0.8980
seed number	0.32	0.18	1.8067	0.0790	-0.02	0.14	-0.17	0.8698	-0.12	0.14	-0.84	0.40470	-0.03	0.14	-0.24	0.8089	0.07	0.10	0.66	0.5147	-0.01	0.15	-0.05	0.9601
(Intercept)	1.78	0.99	1.79	0.0824	-0.78	0.69	-1.14	0.2608	-0.04	0.74	-0.05	0.9627	0.18	0.78	0.24	0.8142	0.85	0.62	1.36	0.1840	0.97	0.82	1.18	0.2465
joint, no abscission	-0.51	0.65	-0.78	0.4411	0.55	0.45	1.23	0.2290	0.46	0.49	0.94	0.3562	0.13	0.51	0.25	0.8026	-0.18	0.35	-0.52	0.6044	0.15	0.54	0.29	0.7761
joint, abscission	-0.65	0.81	-0.80	0.4278	0.16	0.56	0.28	0.7829	1.01	0.61	1.66	0.1069	-0.04	0.64	-0.06	0.9495	-0.32	0.49	-0.65	0.5193	-0.45	0.67	-0.68	0.5042
indehiscent	0.62	0.81	0.76	0.4526	0.67	0.56	1.19	0.2413	-1.26	0.61	-2.07	0.0464	-0.54	0.64	-0.85	0.4027	-1.01	0.41	-2.02	0.0518	-0.23	0.67	-0.34	0.7372
pericarp features	-0.43	0.65	-0.67	0.5086	0.82	0.45	1.83	0.0763	0.78	0.49	1.60	0.1182	0.57	0.51	1.12	0.2698	0.14	0.33	0.44	0.6638	-0.86	0.54	-1.60	0.1183
seed mass (g)	0.44	0.20	2.14	0.0402	-0.27	0.14	-1.88	0.0691	0.22	0.15	1.42	0.1652	-0.12	0.16	-0.76	0.4548	0.05	0.11	0.47	0.6438	0.14	0.17	0.81	0.4252
seed number	0.25	0.27	0.92	0.3653	0.09	0.19	0.50	0.6228	-0.06	0.21	-0.28	0.7847	-0.09	0.21	-0.43	0.6694	-0.15	0.15	-0.98	0.3367	-0.25	0.23	-1.11	0.2736

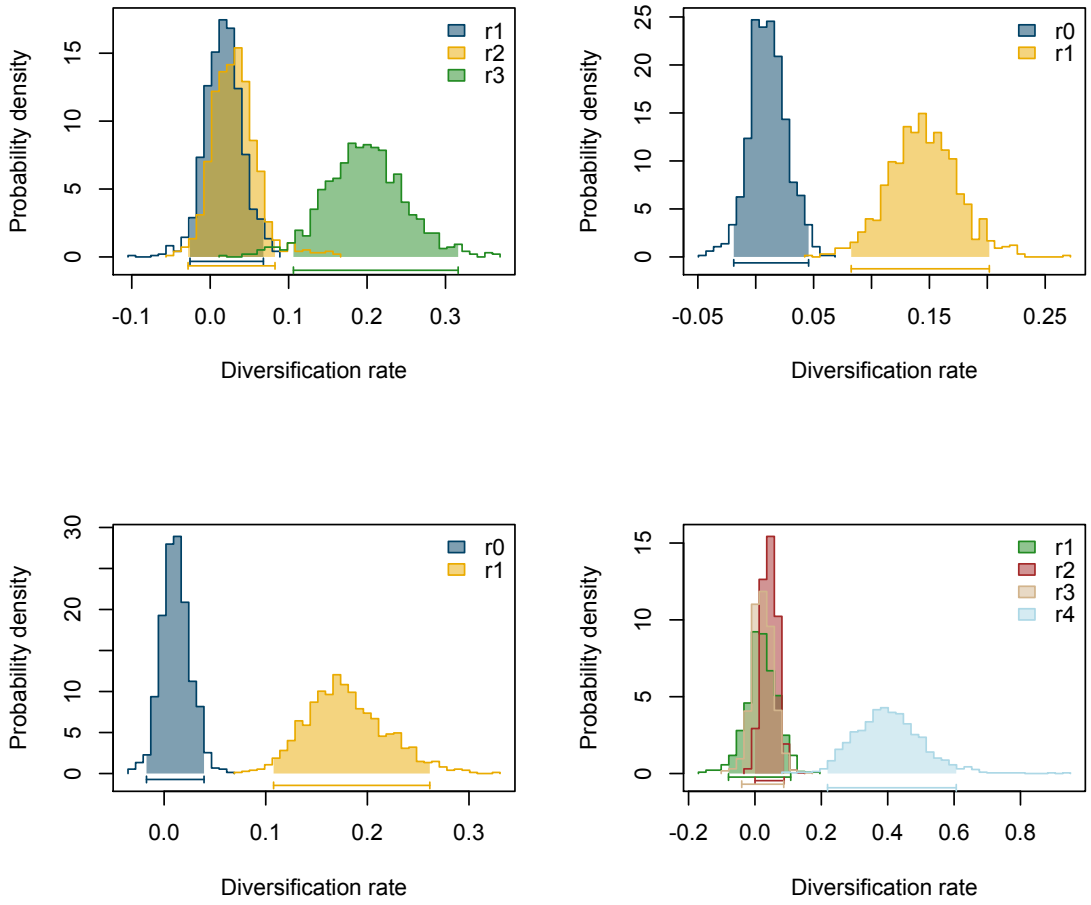


Figure 7. Diversification rates of taxa with different fruit traits and habitat. Diversification rates were calculated as the difference between speciation and extinction rates. Plots are for, from upper right to lower left: Joint (r1 = no joint, r2 = joint, no abscission zone, r3 = joint, abscission zone), indehiscence (r0 = dehiscent, partially dehiscent, r1 = fully indehiscent), pericarp features (r0 = no pericarp features, r1 = pericarp features), and habitat (r1 = field, r2 = ruderal, r3 = desert, r4 = coast). These rate estimates are from diversification analysis on the Bayesian consensus tree as an illustration of the patterns found in a more thorough analysis accounting for unsampled taxa and phylogenetic uncertainty (see Table 9).

2.4 Discussion

This study found evidence that fruit and seed traits coevolve in ways that influence dispersal and establishment. While the evolution of heterocarpy itself did not correspond to changes in dispersal and seed traits, it preceded the

evolution of dispersal-related traits such as abscission, indehiscence, and dispersal-enhancing pericarp features. Second, there was a trend for seed size and/or number to be associated with shifts in habitat and range size. Finally, we found that dispersal-enhancing traits as well as the coastal habitat are associated with significant increases in diversification rate, driven primarily by increased speciation rates. In summary, the evolution of dispersal-enhancing traits appears to result in a series of correlated morphological changes that have significant effects on species establishment ability, niche, and diversification.

Dispersal-related fruit traits in the Brassiceae appear to have strongly co-evolved. Indehiscence, which intuitively might be considered to constrain dispersal, occurred concomitantly with dispersal-enhancing features such as joint abscission, pericarp features, and a reduction of the number of seeds per dispersal propagule. In combination, these traits functionally represent the evolution of enhanced dispersal. Interestingly, traits that promote dispersal (joint abscission and pericarp features) are not correlated themselves. While they can evolve in the same taxa, they appear to be otherwise independent means that permit the evolution of indehiscence without restricting dispersal. Thus, whether or not selection acts directly on dispersal ability, the evolution of these fruit traits generally leads to the evolution of a well-defined set of traits that promote dispersal.

Table 9. Test for significant differences in speciation and extinction rates among taxa with different fruit traits. Differences were tested for by comparing log-likelihoods of BiSSE models in which speciation (λ) and extinction (μ) rates were allowed to vary independently or were constrained to be the same. If the unconstrained model had a significantly larger likelihood (i.e., less likely), then the differences among categories were significant. Significant differences were tested using a χ^2 log-likelihood test. Joint (0 = no joint, 1 = joint), joint + abscission (0 = no joint, 1 = joint, no abscission zone, 2 = joint, abscission zone), indehiscence (0 = dehiscent, partially dehiscent, 1 = fully indehiscent), pericarp features (0 = no pericarp features, 1 = pericarp features), and habitat (0 = field, 1 = ruderal, 2 = desert, 3 = coast). Values represent the median estimate, calculated across all posterior probability distributions from analysis of 100 trees sampled from the phylogenetic Bayesian posterior distribution.

									log-likelihood				
trait	**λ0**	**λ1**	**λ2**	**λ3**	**μ0**	**μ1**	**μ2**	**μ3**	**unconstrained**	**λ constrained**	**P**	**μ constrainted**	**P**
joint	0.04	0.14			0.02	0.01			-244.70	-256.65	0.0013	-252.42	0.0105
joint + abscission	0.03	0.04	0.24		0.01	0.01	0.03		-251.90	-263.32	0.0017	-257.12	0.0368
indehsicence	0.02	0.18			0.03	0.01			-238.04	-245.16	0.0043	-238.04	0.5000
pericarp features	0.02	0.23			0.04	0.01			-239.50	-244.53	0.0144	-236.76	0.1268
habitat	0.05	0.06	0.04	0.48	0.02	0.01	0.02	0.06	-236.90	-255.71	0.0001	-244.55	0.0109

Fruit traits also coevolve with seed mass. Traits associated with enhanced dispersal, i.e., indehiscence plus pericarp features, were also associated with the evolution of larger seeds. Larger seeds have been shown to enhance the probability of seedling establishment. Several studies have demonstrated a positive relationship between seedling establishment and seed size, in species that are animal dispersed (Aizen and Patterson 1990; Edwards and Westoby 1996) and those that are passively dispersed (Moles and Westoby 2004). In addition, large seed mass may be especially important for establishment after long-distance dispersal into novel habitats or unpredictable environments (Cain et al. 2000; Wang and Smith 2002; Moles and Westoby 2004). The enhanced establishment ability of larger seeds after long-distance dispersal is supported by the marginally significant, positive relationship between seed size and latitudinal range size in the tribe. It has been suggested that larger seeds should have wider geographic ranges because they are better able to establish post-dispersal (Edwards and Westoby 1996). The evolution of indehiscence or dispersal-related pericarp features had no direct association with range size. Thus, while these traits might facilitate dispersal, it appears that without the appropriate resources to foster establishment after dispersal, range size will remain static. Therefore selection may favor an association, such as that observed here, between large

seeds and long-distance dispersal in a manner that enhances colonization after dispersal and increases in range size.

The evolution of larger seeds within the tribe was marginally associated with shifts to a coastal habitat, but not to specific climate variables. Larger seed size has been argued to be an adaptation to promote establishment among coastal species, given the limited resource available in most dune systems (Maun 1994). Thus, it is possible that seed size may have increased initially as an adaptation to the coastal environment, rather than in conjunction with fruit traits that enhanced long-distance dispersal.

The evolution of dispersal-enhancing traits was associated with increased rates of diversification within the tribe Brassiceae. The evolution of an abscission zone and pericarp features was associated with elevated rates of diversification, in both cases due to increased speciation rates. This finding is in contrast with some existing theory and empirical evidence. Long-distance dispersal is predicted to limit the process of speciation by increasing gene flow and thereby mitigating adaptive divergence and allopatric isolation (Coyne and Orr 2004; Givnish 2010). In the cases were diversification rates have been compared between clades with and without long-distance dispersal, diversification rates were typically higher in clades without long-distance dispersal (Givnish 2010). However, it is possible that long-distance dispersal might promote speciation by

increasing the likelihood of long-distance colonization events and subsequent allopatric isolation (Cain et al. 2000; Moore and Donoghue 2007). This argument is often invoked within the context of island biogeography and a role of vicariance versus dispersal in driving geographic distributions (Roy et al. 2009). In Brassiceae, this long-distance colonization and allopatry dynamic could be one explanation for the elevated rates of speciation we see associated with dispersal-enhancing fruit traits.

Another explanation for the observed association between increased speciation and traits associated with enhanced dispersal is that long-distance dispersal may facilitate shifts to a novel habitat. In the Brassiceae, dispersal-enhancing traits were indirectly associated, through their association with seeds size, with a shift to the novel coastal habitat, which was in turn associated with increased speciation. The shift to the coast might have opened up new niche opportunities that promoted speciation rates via adaptive radiation. Given the tight co-evolution of these traits, however, it is difficult to disentangle which process has played a dominant role in the diversification of the Brassiceae.

In conclusion, in the Brassiceae, the evolution of enhanced dispersal can occur in multiple ways through the correlated evolution of different fruit traits. Regardless of how it evolves, enhanced dispersal was generally associated with larger seed size, which likely facilitates establishment and for which there is

some evidence of an associated shift in habitat and increase in range size. The combined effect of these evolutionary transitions in dispersal ability and habitat has accelerated the rate of diversification within the tribe. The evolution of increased dispersal, when accompanied with seed features that increase the ability to establish in isolated or novel habitats, can therefore influence macro-evolutionary processes of diversification.

3. Adaptive divergence along a latitudinal gradient of seasonality in the genus *Cakile*

3.1 Introduction

What limits a species' geographic range is a longstanding question in evolutionary ecology (MacArthur 1972; Levin 2000; Holt et al. 2005; Sexton et al. 2009; Gaston 2009). The importance of the climatic environment in defining range boundaries is evident from the close associations between species' borders and specific climatic factors (Geber 2008) and from changes in geographic distribution associated with climate changes (Root et al. 2003; Parmesan and Yohe 2003; Parmesan 2006). Range limits may also be a function of a species' ability to plastically respond or evolutionarily adapt to novel climatic environments at the range border (Kirkpatrick and Barton 1997; Holt 2003; Griffith and Watson 2006).

Adaptation to climatic variation along latitudinal clines is a major determinant of species ranges (Endler 1977). Two major environmental components that vary with latitude and that are thought to define latitudinal range limits are day length and temperature seasonality (Bradshaw et al. 2004). Day length and temperature can act directly as selective agents by establishing the length of the growing season, and they can act indirectly by serving as cues for other seasonal events such as approaching drought or winter.

There is strong evidence that species have undergone adaptive divergence along latitudinal clines. Correlative studies often find significant associations between latitude and ecologically important traits, particularly phenological traits (Masaki 1999; Stenøien et al. 2002; Stinchcombe et al. 2004; Hopkins et al. 2008). Furthermore, studies have found direct evidence for adaptive divergence in response to latitudinally varying climatic factors based on reciprocal transplants, common-garden studies and genetic signatures of selection (Ikeda and Setoguchi 2010; Urbanski et al. 2012; Campitelli and Stinchcombe 2013).

Genetic variation in the ability to inhabit different latitudes can influence latitudinal distribution (Sexton et al. 2009). For instance, the lack of genetic trade-offs in fitness-related traits at the center of the range and the range margins can facilitate range expansion (Holt 2003; Holt et al. 2011). In contrast, genetic trade-offs in fitness-related traits between the center of the range and the margins can limit geographic expansion (Holt 2003; Holt et al. 2011). In general, studies that have tested for such trade-offs using reciprocal transplant experiments have found them (Angert and Schemske 2005; Eckert et al. 2008; Colautti et al. 2010). Genetic variation within species can enable a wide geographic range even if there are trade-offs, depending on the degree of gene flow between populations (Holt et al. 2011). Limited gene flow between marginal populations and center

populations might even promote divergent adaptation and facilitate range expansion (Holt et al. 2011).

The genus *Cakile* (Brassicaceae) consists of several species that grow on the coastal strand throughout the Atlantic, Caribbean, and Mediterranean (Appendix A). Two of these species, *C. edentula* of North America and *C. maritima* of Europe, exhibit wide latitudinal ranges that span over 20 degrees of latitude from Florida and Spain to Newfoundland and Norway, respectively. Within both species, there is genetic and taxonomic evidence for divergence along this latitudinal gradient such that both species have a corresponding northern and southern subspecies (Rodman 1974; Clausing et al. 2000). This parallel divergence into latitudinally differentiated subspecies allows us to test whether divergent adaptation to factors that vary with latitude has occurred in both species.

Here, we investigate whether two species in the genus *Cakile* have undergone parallel adaptive divergence to environmental factors that vary across a latitudinal gradient. We conducted a common garden experiment under controlled growth chamber conditions that mimic the latitudinal gradient of temperature and day length experienced across and beyond the full range of *C. edentula* and *C. maritima*. We ask: 1) Do species respond plastically to these seasonal conditions? 2) Do species exhibit genetic differentiation at the subspecific level in developmental and fitness-related traits in accordance with

latitudinal adaptation? 3) Is the pattern of latitudinal adaptation consistent across species that have diverged on different continents?

3.1 Material and Methods

Taxa and populations. *Cakile* has diversified along a latitudinal gradient in both North America and Europe. The North American species *Cakile edentula* consists of two genetically distinct subspecies, *C. edentula* ssp. *edentula* (CEE) and *C. edentula* ssp. *harperii* (CEH) (Gormally and Donovan 2011). CEE ranges from northern North Carolina to Newfoundland, while CEH ranges from Northern Florida to the Outer Banks of North Carolina (Rodman 1974). They do not appear to hybridize regularly at their range boundary in NC and are presumably kept isolated by the divergent currents localized at the Outer Banks (Rodman 1974). The European species *C. maritima* consists of two genetically distinct regional populations: a Mediterranean group (*C. maritima* Mediterranean or CMM), which can be found throughout the Mediterranean Sea (Clausing et al. 2000; Westberg and Kadereit 2009), and Atlantic group that ranges from southern Spain to Norway and the Baltic Sea. Within the Atlantic group, there is a genetically distinct northern subspecies, *C. maritima* ssp. *baltica* (CMB) (Rodman 1974; Clausing et al. 2000). We therefore used genetically divergent northern and southern populations of each species.

Individuals were collected from representative populations for each of the four taxa. CEE was collected from Point Judith, Rhode Island, USA (41.36, -71.48). CEH was collected from Tybee Island, Georgia, USA (32.01, -80.92). CMM was collected from Tarifa, Spain (36.06, -5.53). CMB was collected from Gdansk, Poland (54.34, 19.05). Seeds from each location were grown under greenhouse conditions for 1-2 generations to minimize maternal effects.

Experimental Design. We conducted a common garden experiment under four simulated seasonal environments in growth chambers. The four environments spanned the latitudinal range experienced by the genus, simulating the temperature and photoperiod conditions of the following sites from north to south: Reykjavik, Iceland (ICE; 64.11, -21.84), Point Judith, Rhode Island (RI; 41.36, -71.48), Tybee Island, Georgia (GA; 32.01, -80.92), and San Salvador, Bahamas (BA; 24.09, -74.44) (Figure 8). RI represented the native environment of CEE and CMB, while GA represented the native environment of CEH and CMM. ICE and BA represented seasonal conditions at or beyond the northern and southern extremes of the two species ranges (Figure 8).

Chamber conditions mimicked the mean temperature and photoperiod changes over the course of a growing season of each site (Figures 8 and 9). The length of the growing season was defined based on the literature (Rodman 1974) and personal observation. Temperature data for the chambers was based on 10-

year monthly averages. For the ICE, RI, and GA chambers, the growing season started at the beginning of the month when average temperatures first rose above freezing. For the BA chamber, the growing season started during the month that began the rainy season. Temperature and photoperiod were changed daily and incrementally from the mean of each month to the next, without diurnal temperature fluctuations.

We grew 1-2 replicates of 8-12 genotypes from each subspecies in each growth chamber. All individuals were germinated under the same conditions (20°C, 12h light/12h dark) in a germination chamber on sterile 1% agar medium. Individuals germinated within 3 days of each other. Seedlings were planted in 6.4 cm square plastic pots with Metro Mix 360 (Hummert International, Earth City, MO, USA). Pots were randomized within each chamber.

Plants were censused every other day for bolting date, i.e., the emergence of the first reproductive apical meristem, a proxy for flowering time (personal observation). Flower number and fruit number were recorded at senescence. Flower number is our best estimate of fitness for *C. maritima*, which is outcrossing (self-incompatible). *C. edentula* is highly autogamous, so fruit number is an accurate measure of fitness for the two subspecies of *C. edentula.* The experiment was repeated in two temporal blocks in two consecutive years (2011, 2012) with the same taxa, genotypes, growth chambers and growth

chamber conditions. Because of unequal replication of genotypes, replicates of each genotype were averaged within years, and genotypic means (within years) were used for the analyses. Bolting date and flower number were recorded for both years, while fruit set was only recorded for the first year. Bolting and flower number were recorded for both years, while fruit set was only recorded for the first year.

To test for divergence and adaptation between sister taxa, we compared measures of life-history and fitness between northern and southern subspecies of *C. edentula* and *C. maritima* across chamber treatments using analysis of variance (ANOVA). Temporal block, chamber condition (ICE, RI, GA, and BA), species (*C. edentula* vs. *C. maritima*), and subspecies (north vs. south) were treated as fixed factors. A significant effect of chamber treatment indicates plasticity to latitudinal factors. All two- and three-way interactions were included in the model when data was available. A significant subspecies effect indicates genetic differentiation between subspecies over all latitudinal treatments. A significant interaction between subspecies and chamber treatment would suggest adaptation if a given population had higher fitness in its native climate than the non-native subspecies. ANOVAs were run in R v. 2.15 (R Core Team 2013).

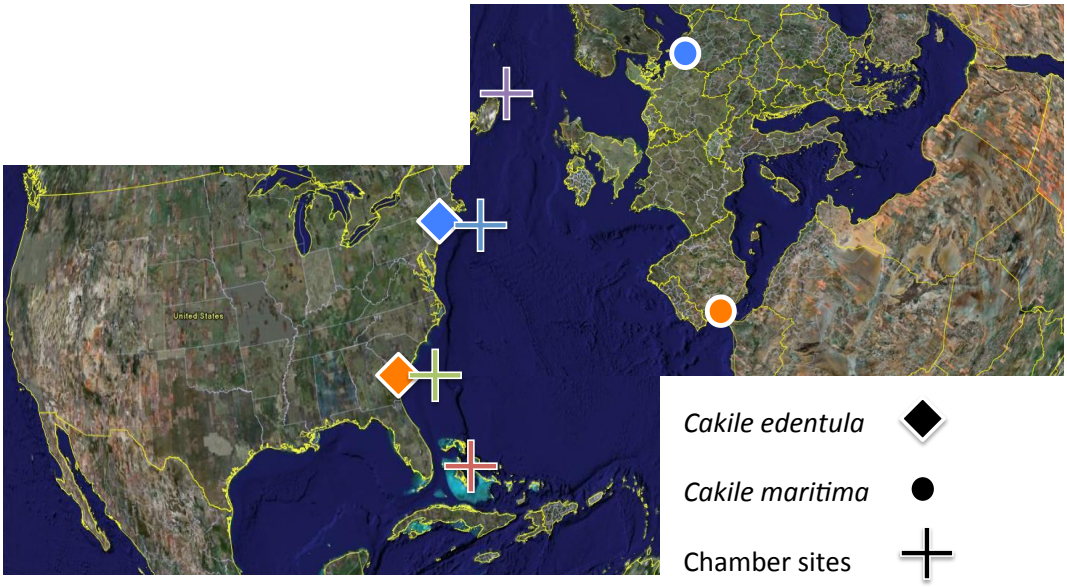


Figure 8. Location of species and chamber sites. Symbols indicated species and location of chamber conditions (see Key). Location of species indicated site where populations were collected (see Material and Methods). Colors for species indicated subspecies populations (orange = southern subspecies, blue = northern subspecies). Colors for chamber locations indicate specific sites and correspond to conditions in Figure 9: Bahamas (red), Georgia (green), Rhode Island (blue), Iceland (violet).

Comparisons between means were tested with Tukey HSD correction for the number of pairwise comparisons. Additional sub-models were performed to interpret interactions. Specifically, each species was analyzed separately.

3.3 Results

Across both species, bolting date, flower number, and fruit set differed significantly across chamber treatment (Tables 10, 11, 12, and 13, Figure 9).

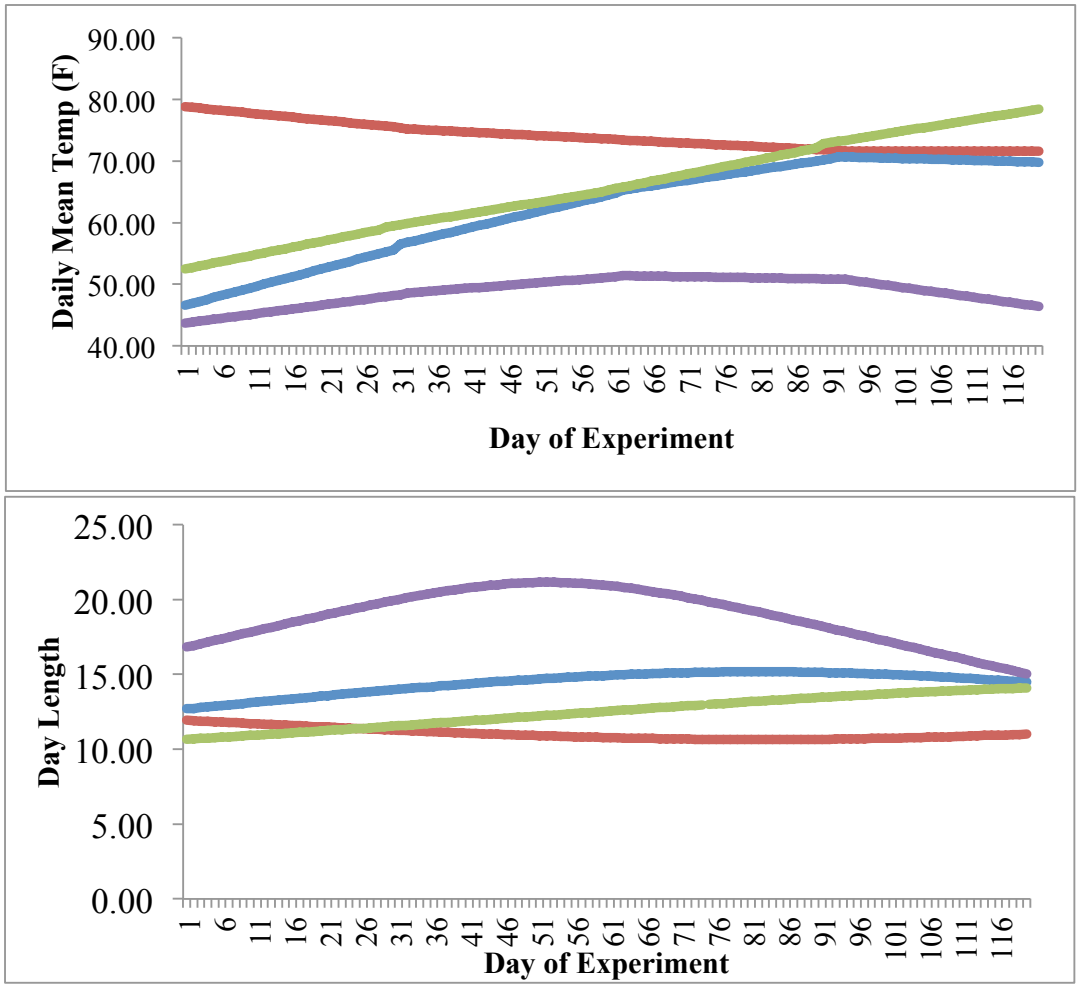


Figure 9. Chamber conditions mimicking latitudinal variation in seasonality. A) Temperature conditions over the course of the experiment. B) Day-length conditions over the course of the experiment. Bahamas (red line), Georgia (green line), Rhode Island (blue line), Iceland (violet line).

This difference was driven mostly by the species' response to the extreme northerly conditions of the Iceland chamber, where plants bolted later, flowered less, and produced fewer fruits (Table 10, Figure 9).

Species differed only slightly in bolting date (Table 10), with *C. maritima* flowering ~1 day later than *C. edentula*, but this difference was not significant

after Tukey HSD correction. Species differed in their overall flower number (*C. edentula,* mean = 19.3, SE = 3.4; *C. maritima,* mean = 94.3, SE = 3.4, Table 12) and fruit set (*C. edentula,* mean = 13.1, SE = 0.7; *C. maritima,* mean = 2.2, SE = 0.7, Table 13), in accord with whether they were selfing or outcrossing. *C. maritima*, an outcrosser, produced more flowers, but fewer fruits than *C. edentula*, a selfer. While a significant species x chamber treatment interaction was detected for all traits (Tables 11, 12, and 13), the response to chamber treatment was qualitatively similar between the species, with later bolting and fewer flowers in the Iceland chamber (Figure 9). *C. edentula* also produced significantly fewer fruits in the Iceland chamber (Figure 9).

Species also differed in the degree of divergence of their subspecies, as indicated by significant interactions between species and subspecies. *C. edentula* had larger differences between subspecies than *C. maritima* for bolting time (Table 10) and fruit number (Table 12), and a significant three-way interaction between species, subspecies, and chamber indicates that the bolting-time responses of the subspecies to chamber conditions also depended on species (Table 10).

Table 10. Means of bolting time, flower number, and fruit number means by species, chamber, year, and subspecies. Species: *Cakile edentula* and *C. maritima.* Chambers: Bahamas (BA), Georgia (GA), Iceland (IC), and Rhode Island (RI). n = sample size. SE = standard error.

				Bolting time			Flower number			Fruit number		
Species	**Chamber**	**Year**	**Subsp**	**n**	**mean**	**SE**	**n**	**mean**	**SE**	**n**	**mean**	**SE**
C. edentula	BA	2011	North	-	-	-	7	17.64	14.03	7	15.17	2.08
C. edentula	BA	2011	South	-	-	-	9	32.83	12.38	9	12.13	1.83
C. maritima	BA	2011	North	-	-	3.59	9	138.94	12.38	9	0.39	1.83
C. maritima	BA	2011	South	-	-	-	8	139.44	13.13	8	0.31	1.94
C. edentula	BA	2012	North	7	33.93	1.91	7	28.41	14.03	-	-	-
C. edentula	BA	2012	South	7	48.48	1.91	8	31.85	13.13	-	-	-
C. maritima	BA	2012	North	4	33.38	2.53	9	69.06	12.38	-	-	-
C. maritima	BA	2012	South	4	46.96	11.47	5	94.70	16.61	-	-	-
C. edentula	GA	2011	North	7	45.50	1.91	7	19.19	14.03	7	17.69	2.08
C. edentula	GA	2011	South	9	63.11	1.69	9	20.24	12.38	9	12.66	1.83
C. maritima	GA	2011	North	9	39.50	1.69	9	193.65	12.38	9	0.65	1.83
C. maritima	GA	2011	South	8	45.71	4.74	8	132.79	13.13	8	0.13	1.94
C. edentula	GA	2012	North	5	49.10	2.26	7	13.99	14.03	-	-	-
C. edentula	GA	2012	South	-	-	-	8	13.72	13.13	-	-	-
C. maritima	GA	2012	North	5	43.60	10.27	10	64.30	11.74	-	-	-
C. maritima	GA	2012	South	1	54.50	18.15	5	85.98	16.61	-	-	-
C. edentula	IC	2011	North	-	-	8.49	7	15.36	14.03	7	6.98	2.08
C. edentula	IC	2011	South	7	67.71	1.91	9	16.44	12.38	9	5.76	1.83
C. maritima	IC	2011	North	5	68.60	2.26	8	54.69	13.13	8	3.58	1.94
C. maritima	IC	2011	South	6	66.50	6.58	8	35.98	13.13	8	5.44	1.94
C. edentula	IC	2012	North	-	-	-	7	2.86	14.03	-	-	-
C. edentula	IC	2012	South	1	53.00	8.30	8	5.04	13.13	-	-	-
C. maritima	IC	2012	North	2	45.50	6.92	9	11.44	12.38	-	-	-
C. maritima	IC	2012	South	4	51.13	2.53	5	16.73	16.61	-	-	-
C. edentula	RI	2011	North	6	47.50	2.06	7	23.55	14.03	7	21.60	2.08
C. edentula	RI	2011	South	9	43.02	1.69	9	32.39	12.38	9	12.85	1.83
C. maritima	RI	2011	North	9	48.85	1.69	9	209.07	12.38	9	1.26	1.83
C. maritima	RI	2011	South	8	47.38	7.52	8	145.85	13.13	8	6.13	1.94
C. edentula	RI	2012	North	2	45.00	10.49	7	11.54	14.03	-	-	-
C. edentula	RI	2012	South	6	52.25	8.21	8	23.97	13.13	-	-	-
C. maritima	RI	2012	North	7	43.38	8.24	10	45.98	11.74	-	-	-
C. maritima	RI	2012	South	5	46.73	10.84	5	71.50	16.61	-	-	-

Table 11. Analysis of variance of bolting date. Species effect compares the difference between *Cakile edentula* and *C. maritima*. Subspecies effect compares the difference between the northern and southern subspecies within *C. edentula* and *C. maritima*. Chamber effect compares the difference among the four latitudinal growth chamber treatments. Year effect accounts for data collected across two replicate years of the study (2011, 2012). DF refers to the degrees of freedom, SS refers to the sum of squares, MSS refers to the mean sum of squares, F refers to the F-value, and P refers to the P-value. Significant effects ($P < 0.05$) are in boldface.

Source	DF	SS	MSS	F	P
Bolting Time					
Species	**1**	**143.80**	**143.79**	**5.62**	**0.0194**
Subspecies (N vs S)	**1**	**2053.00**	**2052.96**	**80.25**	**0.0000**
Chamber	**3**	**5805.10**	**1935.04**	**75.64**	**0.0000**
Year	**1**	**200.90**	**200.95**	**7.86**	**0.0059**
Species X Subspecies	**1**	**175.30**	**175.28**	**6.85**	**0.0100**
Species X Chamber	**3**	**1023.80**	**341.28**	**13.34**	**0.0000**
Subspecies X Chamber	**3**	**905.80**	**301.95**	**11.80**	**0.0000**
Species X Year	**1**	**237.40**	**237.44**	**9.28**	**0.0029**
Subspecies X Year	1	10.10	10.10	0.39	0.5310
Chamber X Year	**2**	**1575.50**	**787.76**	**30.79**	**0.0000**
Species X Subspecies X Chamber	**2**	**274.50**	**137.27**	**5.37**	**0.0059**
Species X Subspecies X Year	1	51.80	51.78	2.02	0.1574
Species X Chamber X Year	2	41.40	20.70	0.81	0.4477
Subspecies X Chamber X Year	2	5.60	2.82	0.11	0.8956
Residuals	118	3018.60	25.58		

Table 12. Analysis of variance of flower number. Species effect compares the difference between *Cakile edentula* and *C. maritima*. Subspecies effect compares the difference between the northern and southern subspecies within *C. edentula* and *C. maritima*. Chamber effect compares the difference among the four latitudinal growth chamber treatments. Year effect accounts for data collected across two replicate years of the study (2011, 2012). DF refers to the degrees of freedom, SS refers to the sum of squares, MSS refers to the mean sum of squares, F refers to the F-value, and P refers to the P-value. Significant effects ($P < 0.05$) are in boldface.

Source	DF	SS	MSS	F	P
Flower Number					
Species	**1**	**371434**	**371434**	**269.41**	**0.0000**
Subspecies (N vs S)	1	203	203	0.15	0.7015
Chamber	**3**	**114373**	**38124**	**27.65**	**0.0000**
Year	**1**	**111584**	**111584**	**80.93**	**0.0000**
Species X Subspecies	1	2011	2011	1.46	0.2285
Species X Chamber	**3**	**67733**	**22578**	**16.38**	**0.0000**
Subspecies X Chamber	3	3156	1052	0.76	0.5159
Species X Year	**1**	**85113**	**85113**	**61.73**	**0.0000**
Subspecies X Year	**1**	**10807**	**10807**	**7.84**	**0.0056**
Chamber x Year	**3**	**20406**	**6802**	**4.93**	**0.0025**
Species X Subspecies X Chamber	3	2222	741	0.54	0.6573
Species X Subspecies X Year	**1**	**12828**	**12828**	**9.30**	**0.0026**
Species X Chamber X Year	**3**	**18869**	**6290**	**4.56**	**0.0040**
Subspecies X Chamber X Year	3	4255	1418	1.03	0.3807
Species X Subspecies X Chamber X Year	3	2882	961	0.70	0.5550
Residuals	217	299177	1379		

Table 13. Analysis of variance of fruit number. Species effect compares the difference between *Cakile edentula* and *C. maritima*. Subspecies effect compares the difference between the northern and southern subspecies within *C. edentula* and *C. maritima*. Chamber effect compares the difference among the four latitudinal growth chamber treatments. Year effect was not included because fruit set was only collected during one of the years. DF refers to the degrees of freedom, SS refers to the sum of squares, MSS refers to the mean sum of squares, F refers to the F-value, and P refers to the P-value. Significant effects ($P < 0.05$) are in boldface.

Source	DF	SS	MSS	F	P
Fruit Number					
Species	**1**	**3714.20**	**3714.20**	**122.96**	**0.0000**
Subspecies (N vs S)	1	60.80	60.80	2.01	0.1588
Chamber	**3**	**370.60**	**123.50**	**4.09**	**0.0085**
Species X Subspecies	**1**	**307.50**	**307.50**	**10.18**	**0.0018**
Species X Chamber	**3**	**842.40**	**280.80**	**9.30**	**0.0000**
Subspecies X Chamber	3	40.70	13.60	0.45	0.7184
Species X Subspecies X Chamber	3	158.50	52.80	1.75	0.1608
Residuals	115	3473.70	30.20		

Because of the interactions between species and subspecies, we tested subspecific differences separately within each species (Table 14, Figure 9). The northern subspecies of both *C. edentula* and *C. maritima*, CEE and CMB respectively, bolted significantly earlier in the southerly chambers (BA and GA) than in the northerly chambers (Table 14, Figure 9). Moreover, northern subspecies bolted earlier than the southern subspecies in the southerly chambers but not in the northerly chambers (Figure 9). Thus northern subspecies are more plastic than southern subspecies in their flowering time response to climatic factors, with greater acceleration of flowering under more southerly conditions.

We found evidence for adaptation to northerly conditions in *C. edentula*. The northern subspecies (CEE) had significantly higher fruit set than the southern subspecies (CEH) when grown under its native northern climate (namely RI; Table 14, Figure 9). However, both subspecies had low fitness in the extreme northerly conditions resembling Iceland (Table 14, Figure 9). Moreover, the southern subspecies did not have higher fitness than the northern subspecies in southerly chambers (Table 14, Figure 9).

In *C. maritima*, no significant differences in fruit set or flower number were detected between northern and southern subspecies in any chamber. Although the southern subspecies did have slightly higher flower number in the most southerly chamber, and the northern subspecies had slightly higher flower

number in the GA and RI chambers, these differences were not even marginally significant (Table 14, Figure 9). As in *C. edentula*, both subspecies had low fitness (flower production) in the extreme northerly chamber (Table 14, Figure 9).

3.4 Discussion

Divergent populations of *Cakile edentula* and *C. maritima* maintained fitness over a wide range of seasonal climates, and only experienced a significant reduction in fitness at the most northern extreme of their latitudinal rage. There was, however, evidence in *C. edentula* that the northern subspecies, *C. edentula* ssp. *edentula*, has undergone adaptive divergence from its southern counterpart, *C. edentula* ssp. *harperii*, and expanded its range to be better adapted to northern climates.

In *Cakile*, two species that have both undergone genetic divergence along a latitudinal gradient provided mixed evidence for adaptive divergence to latitude, despite clear evidence for genetic divergence between southern and northern subspecies (Westberg and Kadereit 2009; Gormally and Donovan 2011). Only one of the species, *C. edentula*, exhibited clear evidence of adaptive divergence to climate. The northern subspecies, *C. edentula* ssp. *edentula*, had higher fitness than its southern counterpart, *C. edentula* ssp. *harperii*, when grown in its native northern climate.

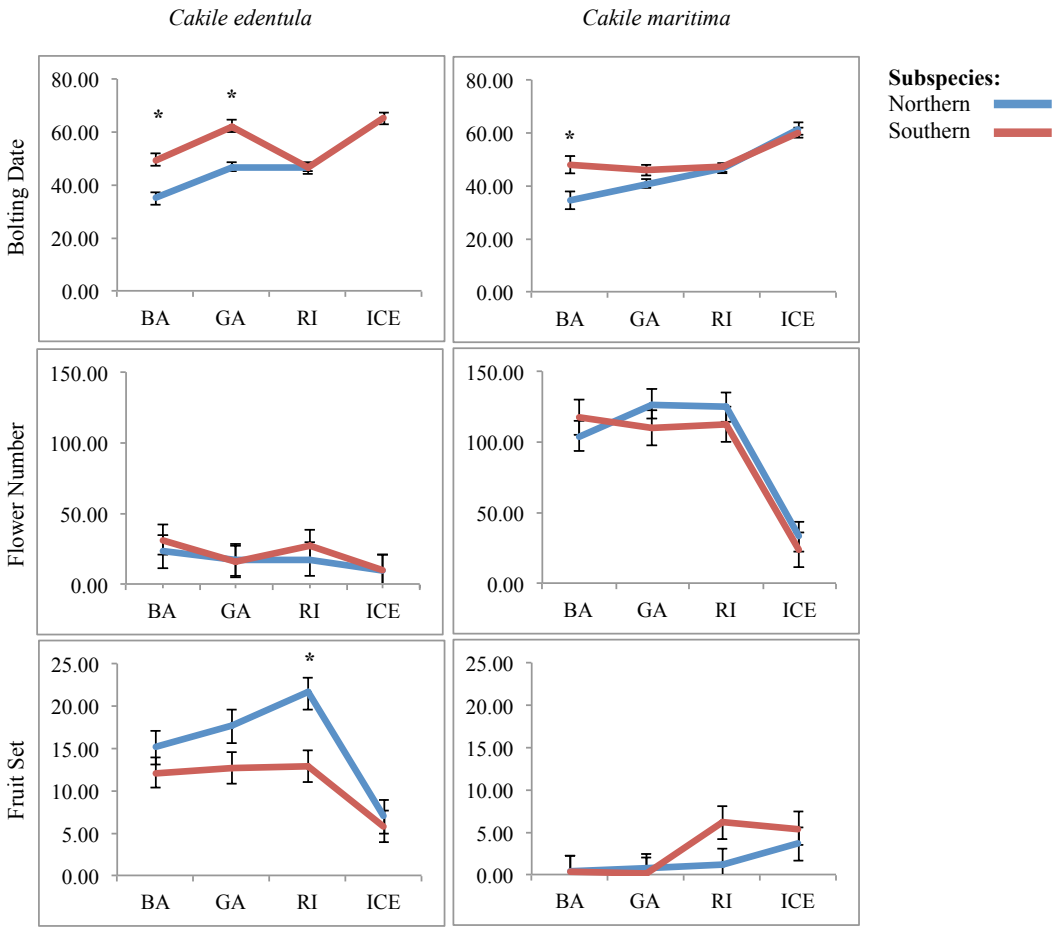


Figure 10. Mean trait values for each subspecies in each chamber. Left column is *Cakile edentula* (North America) with *C. edentula* ssp. *edentula* (northern) in blue and *C. edentula* ssp. *harperii* (southern) in red. Right column is for *Cakile maritima* (Europe) with *C. maritima* ssp. *baltica* (northern) in blue and *C. maritima* ssp. *maritima* (southern) in red. Error bars correspond to standard errors. Chambers include Bahamas (BA), Georgia (GA), Rhode Island (RI), Iceland (ICE). Asterisks indicate significant differences (P < 0.05) based on ANOVA and correction for number of pairwise comparison with a Tukey test.

Table 14. Analysis of variance for the effect of chamber treatment on bolting date, flower number, and fruit set tested across northern and southern subspecies. Chamber reflects the four latitudinal growth chamber treatments. Subspecies is the difference between the northern and southern subspecies within *C. edentula* and *C. maritima*. For bolting date and flower number, year was also included to account for data collected across two replicate years of the study. Fruit set was only collected during one of these years. DF refers to the degrees of freedom, SS refers to the sum of squares, MSS refers to the mean sum of squares, F refers to the F-value, and P refers to the P-value. Significant effects ($P < 0.05$) are in boldface.

		Cakile edentula				*Cakile maritima*			
Source	**DF**	**SS**	**MSS**	**F**	**P**	**SS**	**MSS**	**F**	**P**
Bolting date									
Chamber	3	**2678.48**	**892.83**	**27.99**	**0.0000**	**3676.40**	**1225.47**	**29.24**	**0.0000**
Subspecies (N vs S)	1	**1271.96**	**1271.96**	**39.87**	**0.0000**	133.30	133.29	3.18	0.0790
Year	1	**985.49**	**985.49**	**30.89**	**0.0000**	**701.40**	**701.44**	**16.74**	**0.0001**
Chamber X Subspecies	3	**880.69**	**440.34**	**13.80**	**0.0000**	**351.30**	**117.10**	**2.79**	**0.0468**
Flower number									
Chamber	3	**5710.30**	**1903.45**	**35.58**	**0.0000**	**176042.00**	**58681.00**	**18.72**	**0.0000**
Subspecies (N vs S)	1	**936.10**	**936.09**	**17.50**	**0.0001**	3883.00	3883.00	1.24	0.2680
Year	1	**1131.80**	**1131.82**	**21.16**	**0.0000**	**193133.00**	**193133.00**	**61.61**	**0.0000**
Chamber X Subspecies	3	**616.30**	**205.45**	**3.84**	**0.0116**	4386.00	1462.00	0.47	0.7063
Fruit set									
Chamber	3	**993.14**	**331.05**	**24.17**	**0.0000**	229.22	76.41	1.67	0.1842
Subspecies (N vs S)	1	**320.03**	**320.03**	**23.36**	**0.0000**	38.87	38.87	0.85	0.3611
Chamber X Subspecies	3	**122.87**	**40.96**	**2.99**	**0.0386**	76.35	25.45	0.55	0.6470

This pattern was not seen in *C. maritima*. This could be because the northern subspecies has not adapted well to northerly conditions, or perhaps more plausibly, because our fitness measure of the self-incompatible species was not adequate or the conditions of the growth chambers did not capture all ecologically relevant conditions found at northern latitudes.

The two subspecies of *C. edentula* did not differ in fitness when grown in southern climates and the extreme northern climate, though they both experienced a significant overall reduction in fitness in the northerly extreme. This pattern of adaptive divergence is consistent with the hypothesis that *C. edentula* has undergone adaptive range expansion into the north while maintaining its ability to grow under southern conditions.

While there is evidence of adaptive divergence to latitude, these results also indicate that subspecies of both *C. edentula* and *C. maritima* exhibit a wide range of tolerance to seasonal factors that vary across latitude, since they maintained their fitness across most of the chambers. The only exception is the limited adaptation to the extreme northern environment of Iceland. Thus trade-offs in performance were not detected across the range of conditions experienced across the range of both species. It therefore appears that adaptation to novel latitudes, as with the northward expansion of *C. edentula*, can be accomplished without compromising fitness at lower latitudes. Such lack of tradeoffs can

potentially contribute to the ability of this species to inhabit such a wide latitudinal range.

Unexpectedly, the southern subspecies bolted later than the non-native northern species under southern conditions. Given the unpredictable environment of the coastal strand, we expected species to flower as early as possible relative to their growing season, so as to maximize the likelihood of reproducing before mortality. It is possible, however, that there may be adaptive advantages to delaying reproduction in southern climates such as increased resource accumulation, seasonal changes in pollinator community, or competition, that are not present in northern climates (Griffith and Watson 2005; Donohue et al. 2010; Prendeville et al. 2013). The fact that both southern subspecies have delayed flowering in the southern chambers compared to northern subspecies further suggest that this response might be adaptive. Alternatively, delayed reproduction in the south may not be adaptive, but northern populations may be selected for highly accelerated reproduction, and this response could be most apparent at warmer temperatures because of genotype x environment interaction. Further research must be done determine if delayed flowering time in southern conditions is adaptive.

Whether temperature or day length accounts for the patterns of differentiation and adaptation that we observed cannot be determined from this

experiment. Temperature is known to influence overall developmental rates (Went 1953; Turner and Lahav 1983; Reeves and Coupland 2000; Loveys et al. 2002). The overall reduction in fitness and increased time to flowering in the Iceland chamber is likely driven by reduced temperatures, since plants were not light-limited under long days, but temperatures remained below 10 °C for the majority of the experiment.

Warmer temperatures also likely drove the relatively earlier flowering of northern subspecies in southern climates. Long days are known to induce flowering in many mustard species (Reeves and Coupland 2000; Searle and Coupland 2004), but the southern chambers never attained the maximum day length as the Rhode Island chamber. Therefore, the delayed flowering time of the northern subspecies of *C. edentula* and *C. maritima* is unlikely to be caused by longer days but is more likely to be caused by low temperature. Northern populations may have been selected for faster developmental rates under conditions of shorter growing seasons. Whether this is a passive development response or an active response to a cue remains to be determined. What is more intriguing is what limits the ability of southern subspecies to flower earlier under warmer conditions. Both southern subspecies bolted at approximately the same time after germination in all three chambers from the Bahamas to Rhode Island,

making it difficult to tease apart the relative importance of temperature versus day length.

We found evidence for adaptation to novel northerly conditions encountered during post-glacial range expansion, with no evidence for tradeoffs in fitness across seasonal factors that vary with latitude. What then limits the range of these subspecies and permits genetic divergence across latitude? *Cakile* species and subspecies do exhibit significant genetic differentiation and differentiation in functionally significant traits (Chapter 2, Appendix A). Environmental factors that are not related to differences in temperature and photoperiod across latitude may be more important for such functional trait divergence. The coastal strand is relatively similar across the latitudinal gradient with regard to limited resources, harsh winds and sun, exposure to salt water, soil texture and drainage, etc. Two of the major variables along the latitudinal gradient were addressed in this study: day length and temperature seasonality. However, there are additional differences that could be driving adaptation independently of seasonality in average temperature and day length such as diurnal temperature fluctuations, precipitation, pollinator limitations, herbivory, pathogens, and daily temperature extremes. The variation in these factors across latitudes or their effect of on adaptation in *Cakile* is not known and provides motivation for future reciprocal transplant experiments in the field.

Along with divergent adaptation, dispersal has also likely played a large role in promoting isolation (Chapter 2). There is clear evidence from studies of population structure in *Cakile*, as well as other coastal strand plants, that geographic distance as well as currents play a significant role in promoting genetic divergence (Weising and Freitag 2007; Westberg and Kadereit 2009). Thus subspecies may be genetically and morphologically divergent in part because of neutral divergence accompanying allopatric isolation or isolation by distance.

In conclusion, subspecies of *C. edentula* and *C. maritima* do differ in phenology and other functionally significant traits. Adaptation to northern climatic factors experienced within the current range seems to have occurred in *C. edentula*, but such adaptation does not appear to compromise fitness under certain climatic conditions of southern latitudes. Under conditions of climate change, this northern subspecies may be able to cope with warmer temperatures, provided that this temperature increase does not interact adversely with photoperiod. We did not find this pattern to be shared with *C. maritima*, however, suggesting that this ability to adapt to northerly conditions without fitness tradeoffs in southerly conditions may not be generalizable even to closely related species. Further tests that decouple temperature and photoperiod and tests under field conditions would provide insight into what environmental

factors have contributed to geographic adaptation of both species, and how each might respond to altered climate.

4. Patterns of reproductive isolation in the genus *Cakile* and the role of ecological divergence

4.1 Introduction

Darwin originally argued that the origin of species was driven by divergent selection, whereby new species arose as a consequence of adapting to different environments (Darwin 1859). With the advent of the biological species concept (BSC), the study of speciation was refocused on the evolution of reproductive isolation (RI) as opposed to adaptive differences (Dobzhansky 1937; Mayr 1942; Coyne and Orr 2004). In particular, proponents of the BSC emphasized the evolution of intrinsic post-zygotic incompatibilities (e.g., genic incompatibilities, chromosomal inversions), as the most important component of speciation, a process that does not require divergent selection *per se* (Barton 2001; Unckless and Orr 2009). Thus, the importance of divergent selection in speciation has been seriously questioned (Schluter 2001; Coyne and Orr 2004), and the degree to which divergent selection accelerates the evolution of different components of RI remains controversial (Levin 2000; Schluter 2001; Rundle and Nosil 2005; Hendry et al. 2007; Langerhans et al. 2007; Schluter and Conte 2009).

To understand the role adaptive divergence in the evolution of reproductive isolation, one first needs to characterize reproductive isolation in a given system. Reproductive isolation is the combined product of multiple

barriers that inhibit the successful reproduction of two species (Coyne and Orr 2004). Reproductive barriers are generally categorized into pre-zygotic (before fertilization) and post-zygotic (after fertilization). Within these broader categories are several sequentially acting barriers, the importance of which depends on the order in which they occur as well as how effective they are at inhibiting successful reproduction. Under natural conditions, pre-zygotic barriers (e.g., habitat isolation, phenological isolation, pollinator isolation) have been shown to contribute more to total reproductive isolation since they act earlier in the life cycle and they are less reproductively costly (Ramsey et al. 2003; Lowry et al. 2008a; Widmer et al. 2009). In contrast, post-zygotic barriers (e.g., F_1 viability, F_1 fertility) play a relatively minor role in the cumulative reproductive isolation between adjacent species. However, post-zygotic barriers are considered the most permanent of reproductive barriers because they are difficult to lose once evolved and prevent any subsequent genetic exchange between species even if mating and fertilization has taken place (Orr 1996; Orr and Turelli 2001). There is increasing evidence, moreover, that they provide the impetus for pre-zygotic reproductive isolation to evolve between species undergoing secondary contact (i.e., reinforcement; Servedio and Noor 2003; Smadja and Butlin 2006; Hopkins et al. 2008; Shaw and Mendelson 2013).

One highly informative approach to understanding how reproductive barriers evolve is to evaluate the strength of each barrier in multiple species pairs in a comparative framework (Coyne and Orr 2004). This approach aims to evaluate how rapidly barriers accumulate relative to one another (i.e., their rates of evolution) and in what fashion (linear vs. non-linear). Rate comparisons have typically focused on differences between pre- and post-zyogtic isolation, and have generally found that pre-zygotic barriers evolve faster (Coyne and Orr 1997; Moyle et al. 2004). Few studies have looked at the relative rates of evolution of barriers within pre- or post-zygotic barriers. Additionally, most comparative studies of reproductive isolation detect a positive, linear correlation between genetic distance and the strength of reproductive isolation (Coyne and Orr 1997; Sasa et al. 1998; Price and Bouvier 2002; Mendelson 2003; Jewell et al. 2012), suggesting an approximately "clock-like" accumulation of reproductive isolation. Fewer studies have been able to test for non-linear patterns of the accumulation of RI, which is predicted theoretically to exhibit an exponential, or "snowball", pattern (Orr and Turelli 2001; Turelli et al. 2001). Populations in isolation will accumulate new alleles. As more genetic differences accrue between populations, the lower the probability that each new allele will be compatible with all of the new alleles in the other population. The effect of this process is the exponential increase—or 'snowball'—of the number of

incompatible alleles between populations. These models apply only to the number alleles contributing to reproductive isolation, however, and not the accumulated *magnitude* of reproductive isolation (Orr and Turelli 2001). However, it has been argued that the magnitude of reproductive isolation can be proportional to the number of alleles contributing to it, especially in recently diverged taxa, leading to the expectation that RI itself should increase non-linearly with genetic divergence (Presgraves 2010). If so, then the magnitude of reproductive isolation itself is predicted to increase non-linearly with increased genetic divergence. There is limited, but increasing empirical evidence for such a pattern in plants and animals (Bolnick and Near 2005; Moyle and Nakazato 2010; Matute et al. 2010), though not in yeast (Giraud and Gourbière 2012).

The degree to which divergent selection promotes the evolution of reproductive barriers depends on the barrier in question. Divergent selection, in contrast to parallel selection, is expected to promote the evolution of reproductive barriers that are involved with specific ecological functions in divergent environments. These include several pre-zygotic barriers, such as pollinator isolation and ecological isolation acting through immigrant inviability (Darwin 1859; Nosil and Mooers 2005; Rundle et al. 2005; Hendry et al. 2007; Nosil et al. 2009; Via 2009). These also include disrupted adaptation of hybrids. Collectively, these are referred to as extrinsic post-zygotic barriers (Dobzhansky

1937; Mayr 1942; Coyne and Orr 2004; Levin 2004a; Rundle and Nosil 2005; Nosil et al. 2009). Intrinsic reproductive barriers, in contrast, arise through intrinsic genic incompatibilities in zygotes and are expressed regardless of the ecological context. Whether divergent selection accelerates the evolution of post-mating barriers such as pollen-pistil interaction or barriers that reduce hybrid fitness through intrinsic incompatibilities (i.e., intrinsic post-zygotic barriers) is less clear.

Theoretical models predict that intrinsic barriers will evolve at similar rates under both divergent and parallel selection (Barton 2001; Gavrilets 2004; Unckless and Orr 2009; Conte and Arnegard 2012), provided the alleles that contribute to adaptation are unique to each population. However, intrinsic post-zygotic barriers would evolve more slowly under parallel selection if the same alleles were fixed in both populations, as would be the case if the number of new alleles were limited or if selection operated on similar pools of standing variation (Coyne and Orr 2004; Barrett and Schluter 2008; Schluter 2009; Schluter and Conte 2009). In theory, these same predictions could be extended to the evolution of post-mating pre-zygotic barriers under divergent versus parallel selection; however, this has never been done explicitly.

Empirical studies provide evidence that divergent selection has contributed to reproductive isolation, but such evidence is stronger for some

components of RI than for others. Empirical evidence that divergent selection promotes the evolution of RI comes primarily from studies of species pairs. In plants, most evidence derives from studies of divergent selection on pre-mating barriers (Schluter 2001; Coyne and Orr 2004; Levin 2004b; Rundle et al. 2005; Lowry et al. 2008b; Widmer et al. 2009; Schluter 2009; Moe et al. 2012). Given the importance of pre-mating barriers to the overall RI between species (Ramsey et al. 2003; Coyne and Orr 2004; Martin and Willis 2007), these studies suggest an important role for divergent selection in speciation. Fewer studies have examined how divergent selection has influenced the evolution of post-mating pre-zygotic interactions between species, in particularly pollen-pistil interactions. Pollen-pistil interactions refer to complications with the germination or growth of pollen tubes (i.e., pollen performance) after mating (Swanson et al. 2004). Rather, research on post-mating pre-zygotic barriers has focused on the role of sexual selection (Ramsey et al. 2003; Lowry et al. 2008a; Widmer et al. 2009; Sweigart 2010) and the evolution of selfing (Orr 1995; Orr and Turelli 2001; Brandvain and Haig 2005). However, there is some evidence, primarily from plants, that divergent selection can act either directly or indirectly on traits that affect post-mating pre-zygotic isolation For example, the length the style, a trait often correlated with flower size and other floral display traits and thus a potential target of divergent selection (Fenster and Ritland 1994; Gómez et al.

2009), can significantly impact pollen performance (Delph et al. 1997; Swanson et al. 2004).

Evidence that divergent selection has contributed to post-zygotic reproductive isolation is primarily concerned with the role of extrinsic post-zygotic barriers i.e., environmentally dependent reduction of hybrid fitness caused by disruption of adaptation. In several rigorous experimental field studies, hybrid fitness was found to be either lower in the parental habitats or higher in a habitat that differed from that of the two parents (Wang et al. 1997; Milne et al. 2003; Rieseberg et al. 2003; Coyne and Orr 2004; Gow et al. 2007; Brennan et al. 2009).

Evidence that divergent selection has led to the evolution of intrinsic barriers is rare. Ecological adaptation would contribute to the evolution of intrinsic reproductive barriers if loci underlying intrinsic genic incompatibilities either were pleiotropically or physically linked to loci under direct selection (Coyne and Orr 2004; Rundle and Nosil 2005). Few loci have been found that are known to cause intrinsic genic incompatibilities, (Rieseberg and Blackman 2010; Wolf et al. 2010; Nosil and Schluter 2011), and fewer still have been shown to be linked to other loci under divergent selection (Bomblies and Weigel 2007; Wright et al. 2013). The most concrete example is the evolution of hybrid inviability between divergent populations of *Mimulus guttatus* (Wright et al. 2013). In this

study, the locus associated with hybrid inviability was found to be physically linked to a locus association with copper tolerance that had undergone divergent selection following the colonization of copper mine tailings (Wright et al. 2013). In *Arabidopsis thaliana*, hybrid necrosis has been shown to be link with autoimmune responses that confer disease resistance (Bomblies and Weigel 2007), although variation at this locus may be maintained by balancing selection within populations rather than divergence among populations.

Empirical tests of whether divergent selection promotes the evolution of RI come primarily from studies of species pairs. A comparative framework, however, is an especially powerful tool to test the generality of the role of adaptive divergence in the evolution post-zygotic reproductive isolation. Comparison across multiple species pairs in a phylogenetic context allows one to test for a correlation between adaptive divergence and reproductive isolation while controlling for evolutionary history and divergence time. Funk et al. (2006) used a comparative approach to test if ecological distance, a proxy for adaptive divergence, was correlated with reproductive isolation. They found, generally, a positive correlation between ecological distance and post-zygotic reproductive isolation (pollen inviability) in several taxa, including in Angiosperms. Their results suggest that adaptive divergence may play a role in promoting the evolution of intrinsic post-zygotic reproductive isolation.

The main limitation of Funk et al. (2006) was the coarse measure of ecological distance necessitated by the broad phylogenetic scope of their analysis and limited availability of ecological data on such a broad taxonomic scale. A more focused approach to test whether adaptive divergence promotes the evolution of RI would be to a) comprehensively measure RI within a given system (e.g., a clade, genus, or tribe), and b) quantify specific facets of the environment that have contributed to divergent selection, based on *a priori* evidence, in order to measure ecological divergence among taxa. This type of data, analyzed in a comparative framework, would provide the most accurate means of assessing whether or not ecological divergence contributed to the evolution RI.

The genus *Cakile* Mill. (Brassicaceae) offers an ideal system in which to test whether divergent selection accelerates the evolution of reproductive barriers in general, and intrinsic post-zygotic barriers specifically. The genus is originally derived from desert taxa, but is now primarily restricted to the coastal strand (Rodman 1974; Hall et al. 2006). *Cakile* has nonetheless undergone a recent and extensive diversification into a wide range of climatic habitats (Rodman 1974). The genus ranges from the Arctic to the southern Caribbean and Mediterranean with sister taxa occupying both contrasting and comparable latitudes. There is evidence that sister sub-species have undergone divergent adaptation across this

latitudinal gradient (see Chapter 3, Appendix A). The genus also harbors variation in functional traits, such as leaf and fruit morphology, and biochemical traits, such as gluconsinolate profiles involved with herbivore resistance. Therefore, *Cakile* offers a system with interesting diversification and evidence for adaptive divergence, making it highly suitable for an analysis of the contribution of adaptive differentiation to the evolution of reproductive isolation.

To test whether divergent selection promotes the evolution of intrinsic post-zygotic RI, we characterized the evolution of reproductive isolation across the genus *Cakile* and the allied genus *Erucaria* in relation to ecological distance. First, to determine which components of RI are strongest and have evolved fastest, we compare the strength and rate of accumulation of RI for different components of post-zygotic reproductive barriers. Second, to determine if measures of RI conformed to the expectations of the 'snowball' model, we tested for non-linear accumulation of reproductive isolation. Third, to determine if adaptive divergence promoted the evolution of RI, we tested whether different components of reproductive isolation are associated with ecological distance, after accounting for genetic distance.

4.2 Material and Methods

The study system and crossing design. *Erucaria* and *Cakile* are sister genera in the family Brassicaceae, tribe Brassiceae (Beilstein et al. 2006; Hall et al. 2011; Al-

Shehbaz 2012). The set of taxa studied here include three species of the genus *Erucaria* and 15 species, sub-species, and regional variants of the genus *Cakile*. This includes 11 out of the 13 recognized species and sub-species within *Cakile*, and 3 of the 10 species within *Erucaria*. See Table 15 for a complete list of taxa and their locations.

For each taxon, 2-3 replicates from 1-6 families (i.e., genotypes; median = 4) were grown in the Harvard University Glasshouse under a 12 hour photoperiod, supplemented with mercury-halogen lamps in the evening or cloudy days. Temperatures were set for an average of 25 °C and ranged from 23-30 °C. Plants were watered every other day as needed. Experimental reciprocal crosses were performed between randomly selected families for all 18 taxa combinations (Table 16). All crosses were performed from Oct. 2006 – Jan. 2007. To prevent self-pollination, flowers selected for crossing were emasculated in-bud prior to anthesis, the night before the cross. Crosses were performed the following morning by hand. A total of 3182 crosses were performed with a median of 19 crosses per taxon pair (range = 3 – 35). There were, on average, 4 families per taxon, there were approximately 16 unique combinations between the families of two taxa (and 32 reciprocal combinations). To better capture the full genetic variation of reproductive isolation between two taxa, we completed as many of these unique, but fully reciprocal cross combinations as possible. On

average, we completed ~10 (median cross number, 19, divided by 2, to account for reciprocal crosses) out of 16 of these combinations. When possible, we replicated crosses between unique family combinations. Only 2-3 crosses to the same individual maternal plant were performed on the same day so as to minimize the impact of differential resource allocation on cross success.

Measures of reproductive isolation barriers. From these crosses, the following reproductive barriers were measured under benign greenhouse conditions: F_1 viability measures included cross failure, seed germinability, and survival to flower. F_1 fecundity was measured as flower number and fruit set.

Cross failure was scored as the ability to produce fully developed fruit and seed following hand-pollination (Table 17). Cross failure can be the result of both pollen-pistil interactions (post-mating, pre-zygotic) and embryo inviability (post-zygotic). A subsequent study using a subset of taxa found significant evidence for pollen-pistil interactions, but these interactions did not ultimately prevent the pollen tubes from reaching the ovule, given pollen and time (personal observation). Therefore, we treated cross failure as a measure of F_1 viability.

Seeds produced from the above crosses were used in subsequent measures of F_1 viability and fertility (Table 17). To achieve sufficient sample sizes for each measure of F_1 viability and fertility, we grew two F_1's populations, one

in 2007 and 2008. For each of these two 'time blocks', we divided the greenhouse into 3 spatial blocks. We attempted to grow 1-3 replicates per cross within each of the 6 blocks (2 time, 3 spatial); however, there was not always enough available seed or surviving seedlings to estimate all fitness components for all crosses.

Seed germinability has dramatic fitness consequences and suggests development incompatibilities related to seed viability. Germinability was scored as the ability of seeds to germinate under standard growth-chamber conditions (12/12 day length, 20 °C). Since we were interested in overall germinability, as opposed to germination time, seeds were scarified to promote germination. Seeds were then plated on a 1% agar medium. Germinability was scored in this way for two of the three blocks in 2007 and for all blocks in 2008. In both cases, germination occurred quickly (within 3-6 days of plating), or not at all.

For both time blocks, after 8 days, all germinants were transferred from the growth chamber to the greenhouse. Plants were potted in a 50/50 mixture of Turface (Turface Athletics, Buffalo Grove, IL, USA) and Metro-mix 360 potting soil (Hummert International, Earth City, MO, USA) in 7.6 cm diameter terra cotta pots. Plants were treated as needed for thrips with 1% granular Marathon (OHP Inc., Mainland, PA, USA). Plants were followed from germination to senescence,

or the end of 6 months (at which point > 90% were dead). After senescence, or at the end of 6 months, plants were harvested.

Survival to flowering, i.e., the ability to reach reproductive maturity, has important fitness consequences given the annual habit of the species in this study. Failure to flower yields zero reproductive output. Survival to flowering was scored as the ability of a plant to flower before death. Fecundity was measured as the total number of flowers and fruits on harvested plants. Measures of fecundity have multiple fitness implications. Most directly, fruit number is proportional to the number of progeny contributing to the next generation. Flower number is an indirect measure of number of offspring, in that it represents the opportunity for pollination and potential fruit set. Flower number is also an important measure of floral display, which in turn, has implications for pollination success and fruit set, especially among self-incompatible species (Barrett 2003). Some species in this genus are highly autogamous, while others are highly outcrossing and exhibit self-incompatibility. For selfing plants, fruit set is an accurate measure of total fitness, but for self-incompatible plants, flower number may be a more accurate indicator of fitness. A further complication is that fruit set in selfers may reduce subsequent flower production, making it difficult to compare flower number across selfing and outcrossing plants. To test this possibility, hand pollinations were conducted on

a subset of outcrossing taxa: *C. maritima* (Spain), *C. maritima* (Poland), *C. lanceolata* (Bahamas), *C. arctica* (Iceland). For each species within each treatment there were 4-8 genotypes with 1-2 replicates. Open flowers were pollinated with pollen from different individuals within species every two days. Flower number was recorded on those that received pollen and those that did not. Genotypic variation was not accounted for, and replicates were averaged within genotype. Hand pollination did/did not have a significant effect on flower production in these taxa (two-way ANOVA: Pollination treatment, DF = 1, F = 0.01, P = 0.9774; Species, DF = 4, F = 0.21, P = 0.9317; Treatment X Species, DF = 4, F = 0.39, P = 0.8176), suggesting that comparisons of flower production across taxa may be valid.

For each stage of reproductive isolation, reproductive isolation was assessed relative to intraspecific fitness components: reproductive isolation = 1 – ((average success of interspecific cross)/(average success of intraspecific cross)). In certain instances, interspecific success was greater than intraspecific success (heterosis), resulting in a negative RI value. These values were not removed. All measures of reproductive isolation can therefore range between -∞ to 1 (complete reproductive isolation). With the exception of flower number, there were few instances of values < 0. Measures of hybrid viability and fertility occur in sequential order over the lifetime of a plant. The degree of reproductive isolation

therefore effectively accrues with each subsequent stage. We estimated the absolute contribution and total reproductive isolation as described in Ramsey et al. (2003) for each of the three major stages of post-zygotic reproductive isolation: cross failure, viability (germination + survival to flower), and fecundity (flower number + fruit set). We also calculated two measures of total post-zygotic reproductive isolation. $RI_{Total\ survival}$ combines cross failure and viability. $RI_{Total\ Fitness}$ combines cross failure, viability, and fecundity.

Genetic distance and phylogeny. To estimate genetic distance independently of phylogeny, we used inter-simple sequence repeats (ISSRs) (Wolfe 2005). ISSRs are preferable to other fingerprinting markers because they are highly variable, comparatively reproducible, inexpensive, and easy. They produce a genomic finger-print of dominant markers. The ISSR primers and methods used in this study were taken from Clausing et al. (2000). From five ISSR primer sets, we scored 45 unique characters across all 18 taxa. Genetic distance was calculated using the method from Nei & Li (1979).

Table 15. List of taxa and locations.

species	Species Code	location/town	country
Cakile arabica	ARAB	Medina	Saudi Arabia
C. arctica	TRUM	-	Iceland
C. constricta	BS	Gulf Shores, AL	USA
C. edentula edentula	CM	Cape May, NJ	USA
C. edentula edentula	PJ	Point Judith, RI	USA
C. edentula harperii	TI	Tybee Island	USA
C. edentula lacustris	LUD	MI	USA
C. edentula lacustris	MB	Mount Baldi, MI	USA
C. lanceolata fusiformis	NPB	Lake Worth, FL	USA
C. lanceolata lanceolata	BER	Bermuda	United Kingdom
C. lanceolata lanceolata	BG	Boca Grande Key, FL	USA
C. lanceolata lanceolata	UI	Union Island	Grenadines
C. lanceolata psuedoconstricta	MC	Martin County, FL	USA
C. maritima	1396	Pontevedra	Spain
C. maritima	CA	Carmel, CA	USA
Eurcaria erucarioides	1944	Bechar	Algeria
E. hispanica	2055	Leeds	United Kingdom
E. ollivieri	2983	Agadir	Morocco

Table 16. F_1 cross matrix. Numbers in the matrix represent number of crosses between taxa pairs ("cross pairs"). See Table 15 for species codes. Rows represent the maternal recipient, while columns represent the paternal donor. The lower and upper matrix reflects differences in reciprocal cross numbers. The diagonal represents crosses within taxa. F_1 survival and fitness measures were later averaged across reciprocal hybrids.

	1396	1944	2055	2983	ARAB	BER	BG	BS	CAR	CM	LUD	MB	MC	NPB	PJ	TI	TRUM	UI
1396	4	8	6	7	8	7	5	4	7	7	5	5	8	5	3	7	5	7
1944	4	9	9	7	8	7	8	8	8	8	4	7	6	7	7	4	6	6
2055	1	1	4	3	2	5	6	7	7	4	4	7	6	6	4	6	4	5
2983	6	3	4	6	9	11	9	10	8	8	10	7	11	8	13	11	11	9
ARAB	7	3	12	13	11	17	11	15	12	5	13	14	12	11	9	8	13	5
BER	10	1	6	5	13	5	17	18	14	14	10	10	12	10	12	13	9	18
BG	11	7	9	11	19	16	12	17	14	13	17	16	18	16	14	17	16	13
BS	16	12	13	14	18	17	18	16	16	14	14	16	15	16	15	8	17	14
CAR	9	10	13	15	12	15	17	13	10	16	12	13	12	14	15	13	14	15
CM	4	4	4	4	6	12	6	10	9	3	7	7	9	7	6	12	12	8
LUD	6	5	7	5	6	8	12	14	10	9	7	10	8	9	10	11	11	10
MB	6	10	12	14	9	10	9	12	11	7	12	5	7	7	9	8	8	9
MC	8	8	7	9	12	9	8	9	10	11	9	8	10	11	13	10	12	8
NPB	8	4	8	4	11	9	12	13	9	10	11	11	8	5	5	9	10	8
PJ	7	5	6	6	12	10	12	11	13	12	7	8	10	7	7	11	12	10
TI	14	9	13	9	19	14	15	15	18	9	17	10	13	9	10	4	9	10
TRUM	12	5	8	6	11	10	16	16	11	10	10	10	15	10	7	11	7	8
UI	13	7	10	7	14	13	18	12	14	12	12	13	13	11	13	13	16	9

For the purposes of comparative analysis, we constructed a phylogeny for all 18 taxa. Four markers, two nuclear (*ITS1-ITS4, Fnr*) and two chloroplast (*psbA-trnH, Bras4-trnG*), were sampled for each taxon. These four markers were sampled in addition two markers (nrDNA: *phyA* and cpDNA: *matK)* previously sequenced for the same set of taxa by Hall et al. (2011). These markers were combined and analyzed using maximum likelihood methods, with different models allowed for each gene partition (Posada 2008), and implement in Garli 2.0 (Zwickl 2006) on CIPRES v 3.2 (Miller et al. 2010). Details of this analysis are provided in Chapter 2.

Measures of ecological distance. *Functional Trait Distances.* Similarity of functional traits was calculated based on measurements of functional leaf morphology. Leaf area and perimeter were measured for all 18 taxa grown in the greenhouse. These measurements were used to calculate 'leaf lobedness' (perimeter/area), a functional trait associated with temperature tolerance and thermoregulation (Sack et al. 2003). In the *Cakile* clade, leaf lobedness is also significantly associated with precipitation, suggesting that its variation has adaptive significance (Appendix A). Similarity of functional traits was calculated as the Euclidean distance between the trait means (Table 18).

Glucosinolates are secondary compounds commonly associated with plant defense against local herbivores and have been shown to be under strong

selection (Mauricio and Rausher 1997; Benderoth et al. 2006). Glucosinolate profiles were taken from the literature based on a study by Rodman (1974). Data were available for all 14 *Cakile* taxa, excluding *C. arabica.* The concentration of 17 glucosinolate compounds was estimated for each of these 14 taxa. In the *Cakile* clade, glucosinolate profiles differ significantly across taxa and are correlated with measures of precipitation, suggesting that variation in glucosinolates has adaptive significance. The overall similarity of glucosinolate profiles among taxa was calculated based on the combined Euclidean distance across all 17 compounds (Appendix A, Table 18).

Geographic distance. We measured geographic distance between taxa based on the mean latitude and longitude from distribution data (Table 19). Geographic distance was calculated as the linear distance between the location means on a geodesic-surface (http://www.nhc.noaa.gov/gccalc.shtml).

Climatic Niche Overlap. We estimated climatic niche envelops (CNEs) for all 18 taxa using the software MaxEnt v3.3.3k (Phillips et al. 2006; Phillips and Dudik 2008). Niche overlap is independent of geographic overlap or distance. MaxEnt uses geo-coordinate and climatic data to generate a probability distribution of species occurrence over a given area using the principle of maximum entropy (Guisan and Thuiller 2005). Geo-coordinate data were obtained from a combination of the GBIF data portal, literature sources, and personal

observations of occurrence of each species. For our climatic inputs, we used the 19 bioclimatic variables from the WorldClim Global Data set + altitude at a resolution of arc 2.5′ (~5 km^2) (Hijmans et al. 2005). Species CNEs estimates and projected distributions were restricted to the current, native range of taxa included in this study (North America, Europe, North Africa, and the Near East). Projected distributions were used to calculate niche overlap (Schoener's D)—a proxy for niche similarity—between taxa using the 'phyloclim' package (Evans et al. 2009) in R v. 2.15 (R Core Team 2013).

Climatic Distances. To investigate which climatic factors might be associated with reproductive isolation, climate data for each taxon were extracted based on geo-coordinate data (Table 19). Principle component analysis of these data was performed in R to determine the major components of variation in climate across the taxa group. The first two principle components explained 98.7 % of the variance in the data (PC1 = 94.1%, PC2 = 4.6%). The major loading of PC1 was temperature seasonality (annual standard deviation of monthly temperatures, loading score = 0.995). The major loading of PC2 was annual precipitation (loading score = 0.788). PC differences between taxa pairs were calculated as the Euclidean distance.

Statistical analysis. *Rates of evolution of different components of RI.* The relative "rates" of evolution were estimated using multiple statistical methods. First,

based on Coyne and Orr (1989; 1997), we divided each stage of RI into 4 levels based on the degree of RI (≤0.25, ≤0.5, ≤0.75, ≤1). For each RI component, we then compared the mean genetic distance across the four levels of RI with both a one-way ANOVA and Mann-Whitney U-Test.

To test if reproductive isolation increased with genetic distance, a proxy for divergence time, we tested for correlations between genetic distance and RI for each component of RI. We also tested for correlations between genetic distance versus geographic and ecological distance.

This sort of analysis poses a challenge because of the pairwise structure of the data. Not only are the same taxa used for multiple crosses, but these taxa are also structured differentially by their shared evolutionary history. To correct for the phylogenetic non-independence of these data, phylogenetic independent contrasts are calculated (PICs; Felsenstein 1985). PICs average the measures of reproductive isolation (or ecological distance) across descendent nodes, weighted by branch lengths (Fitzpatrick 2002). The limitations of PICs, however, are twofold: first, there is not always sufficient phylogenetic information available (Tiffin et al. 2001; Moyle et al. 2004), and second, they greatly reduce the dataset, limiting the ability to detect significant statistical relationships.

A variety of statistical methods have been used to analyze both uncorrected and phylogenetically corrected data. The most appropriate analysis

for pairwise data is a Mantel or partial-Mantel permutation test. Most studies, however, lack a complete dataset of pairwise comparisons necessary for a proper Mantel test. Instead, studies have used both standard parametric (linear regression, analysis of variance) and non-parametric statistics (kendall's tau, Mann-Whitney U-test).

To test for correlations between RI stages and genetic distance, we took a comprehensive approach, and used both standard parametric (linear regression) and non-parametric methods (Kendall's tau), applied to both phylogenetically corrected and uncorrected data. We did not perform Mantel tests because several of the cross pairs were not represented in the dataset, especially for later stages of RI.

For phylogenetically corrected data, we used methods outlined in Fitzpatrick (2002). Measures of reproductive isolation and genetic distance were averaged across descendent nodes and weighted by branch lengths. As noted above, we used a different set of markers to construct the phylogeny than we used to calculate genetic distance. Unfortunately, there was limited resolution from our phylogenetic analysis. To account for this phylogenetic uncertainty, we generated a set of 100 trees, where nodes with <70% bootstrap support were randomly resolved. We then calculated PICs and PIC correlations across this set

of trees. Here, we report the median and 95% CI for each correlation coefficient and corresponding p-value.

Tests for non-linear increases in RI over time. In addition to testing for a linear relationship between reproductive isolation and genetic distance, we also evaluated whether reproductive isolation accumulated in a non-linear fashion (Orr and Turelli 2001). In contrast to a steady increase in reproductive isolation, it has been proposed that post-zygotic reproductive isolation, underpinned by genic incompatibilities, would evolve in a non-linear pattern ("snowball prediction") (Orr and Turelli 2001). To test whether reproductive isolation accumulates in a non-linear fashion, we compared the relative fit of a linear model ($y = ax$) to two quadratic models ($y = ax + bx^2$; $y = ax^2$). We evaluated both nonlinear models because the exact form of nonlinear accumulation can vary under different models of genic interactions (Moyle et al. 2010) Models were compared with the Akaike Information Criterion (AIC).

Contributions of ecological distance to different measures of RI. To test if ecological divergence was associated with RI, we included ecological distance as a covariate with genetic distance in both our uncorrected and phylogenetically corrected linear models, as described above. Including genetic distance controls for the effect of divergence time between species, and allows us to directly test the effect of ecological divergence on the accumulation of RI.

4.3 Results

Magnitude of reproductive isolation. Significant reproductive isolation reproductive isolation (RI) was detected among taxa, with means ranging from 0 to 27% for different components of RI, and complete RI being attained among some pairs of taxa for each measure (Table 20). Cross failure, seed germinability, and survival to flower exhibited similar levels of reproductive isolation to each other. In contrast, the reproductive isolation expressed at the later stages of F_1 fecundity was notably weaker (Table 20). Cumulative RI based on total fitness was 0.53 on average, and ranged from -0.81 (heterosis) to 1 (complete RI).

Evolutionary rates of reproductive isolation. Comparison of evolutionary rates across stages of reproductive isolation found that cross failure had an overall slower rate of evolution than later stages of intrinsic post-zygotic RI (Table 21, Figure 11), even though it ultimately reached the highest level of all stages. Seed germinability, survival to flowering, flower number, and fruit number all evolved at similar rates (Table 21, Figure 11).

Table 17. F_1 means for measures of survival and fitness. Cross Pairs can be referenced in cross matrix (Table 16). Parents of each cross pair are included here. n = sample size. SE = standard error.

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
1	1396	1396	4	1.00	0.00	10	0.90	0.10	5	1.00	0.00	6	5.69	0.17	6	2.40	0.47
2	1944	1396	12	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-	-
3	2055	1396	7	0.14	0.14	-	-	-	-	-	-	-	-	-	-	-	-
4	2983	1396	13	0.38	0.14	3	0.33	0.33	2	0.00	0.00	2	4.52	0.47	2	2.46	2.46
5	ARAB	1396	15	0.33	0.13	3	0.33	0.33	1	1.00	-	1	5.36	-	1	1.79	-
6	BER	1396	17	0.71	0.11	9	0.89	0.11	6	0.83	0.17	7	5.57	0.34	7	2.48	0.44
7	BG	1396	16	0.75	0.11	12	0.83	0.11	9	0.78	0.15	10	5.81	0.15	10	1.33	0.48
8	BS	1396	20	0.90	0.07	12	0.58	0.15	7	1.00	0.00	7	5.80	0.31	7	4.70	0.37
9	CAR	1396	16	0.88	0.09	6	0.83	0.17	4	1.00	0.00	7	5.44	0.50	7	2.97	0.31
10	CM	1396	11	0.45	0.16	3	1.00	0.00	-	-	-	-	-	-	-	-	-
11	LUD	1396	11	0.55	0.16	3	0.33	0.33	1	0.00	-	1	6.95	-	1	1.10	-
12	MB	1396	11	0.55	0.16	3	0.33	0.33	1	1.00	-	1	5.66	-	1	0.69	-
13	NPB	1396	16	0.50	0.13	2	0.50	0.50	1	0.00	-	-	-	-	-	-	-
14	NPB	1396	13	0.46	0.14	6	0.50	0.22	3	1.00	0.00	2	6.15	0.45	2	0.74	0.35
15	PJ	1396	10	0.60	0.16	3	0.33	0.33	-	-	-	2	5.54	0.10	2	3.02	0.54
16	TI	1396	21	0.57	0.11	11	0.82	0.12	8	1.00	0.00	8	5.77	0.15	8	4.35	0.26
17	TRUM	1396	17	0.71	0.11	9	0.44	0.18	3	1.00	0.00	2	6.03	0.33	2	2.04	0.43
18	UI	1396	20	0.50	0.11	9	0.44	0.18	3	1.00	0.00	3	5.76	0.25	3	3.70	1.12
19	1944	1944	9	0.89	0.11	15	0.93	0.07	1	1.00	-	1	5.06	-	1	5.68	-
20	2055	1944	10	0.10	0.10	-	-	-	-	-	-	-	-	-	-	-	-

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
21	2983	1944	10	0.10	0.10	-	-	-	-	-	-	-	-	-	-	-	-
22	ARAB	1944	11	0.09	0.09	-	-	-	-	-	-	-	-	-	-	-	-
23	BER	1944	8	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-	-
24	BG	1944	15	0.60	0.13	4	0.25	0.25	-	-	-	1	5.74	-	1	0.40	-
25	BS	1944	20	0.65	0.11	7	0.00	0.00	-	-	-	1	5.75	-	1	2.96	-
26	CAR	1944	18	0.11	0.08	-	-	-	-	-	-	-	-	-	-	-	-
27	CM	1944	12	0.25	0.13	-	-	-	-	-	-	-	-	-	-	-	-
28	LUD	1944	9	0.67	0.17	3	0.33	0.33	1	1.00	-	2	4.84	0.98	2	5.70	0.91
29	MB	1944	17	0.53	0.12	6	0.17	0.17	1	1.00	-	1	5.83	-	1	2.77	-
30	MC	1944	14	0.07	0.07	-	-	-	-	-	-	-	-	-	-	-	-
31	NPB	1944	11	0.36	0.15	-	-	-	-	-	-	-	-	-	-	-	-
32	PJ	1944	12	0.33	0.14	1	1.00	NA	1	0.00	-	1	3.53	-	1	5.04	-
33	TI	1944	13	0.54	0.14	3	0.00	0.00	1	0.00	-	-	-	-	-	-	-
34	TRUM	1944	11	0.36	0.15	-	-	-	-	-	-	-	-	-	-	-	-
35	UI	1944	13	0.23	0.12	-	-	-	-	-	-	-	-	-	-	-	-
36	2055	2055	4	1.00	0.00	11	0.91	0.09	6	1.00	0.00	6	5.23	0.30	6	2.81	0.85
37	2983	2055	7	0.29	0.18	-	-	-	-	-	-	-	-	-	-	-	-
38	ARAB	2055	14	0.50	0.14	4	0.00	0.00	-	-	-	-	-	-	-	-	-
39	BER	2055	11	0.55	0.16	3	0.00	0.00	-	-	-	-	-	-	-	-	-
40	BG	2055	15	0.53	0.14	3	0.33	0.33	2	0.50	0.50	1	6.18	-	1	0.00	-
41	BS	2055	20	0.80	0.09	8	0.13	0.13	1	1.00	-	4	4.86	0.56	4	5.18	0.28
42	CAR	2055	20	0.40	0.11	6	0.17	0.17	-	-	-	-	-	-	-	-	-
43	CM	2055	8	0.50	0.19	-	-	-	-	-	-	-	-	-	-	-	-
44	LUD	2055	11	0.55	0.16	3	0.33	0.33	1	1.00	-	1	2.65	-	1	4.66	-
45	MB	2055	19	0.63	0.11	6	0.17	0.17	2	0.50	0.50	1	6.07	-	1	0.00	-
46	MC	2055	13	0.38	0.15	2	0.00	0.00	-	-	-	-	-	-	-	-	-

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
47	NPB	2055	14	0.79	0.10	7	0.29	0.18	1	1.00	-	1	6.30	-	1	1.09	-
48	PJ	2055	10	0.60	0.16	3	0.00	0.00	-	-	-	-	-	-	-	-	-
49	TI	2055	19	0.58	0.12	6	0.00	0.00	-	-	-	-	-	-	-	-	-
50	TRUM	2055	12	0.50	0.16	3	0.00	0.00	-	-	-	-	-	-	-	-	-
51	UI	2055	15	0.40	0.13	3	0.00	0.00	-	-	-	-	-	-	-	-	-
52	2983	2983	6	0.83	0.17	12	0.92	0.08	8	1.00	0.00	7	5.22	0.27	7	3.07	0.56
53	ARAB	2983	22	0.64	0.11	12	0.58	0.15	6	0.83	0.17	4	6.17	0.12	4	1.51	0.77
54	BER	2983	16	0.88	0.07	12	0.75	0.13	8	0.88	0.13	9	6.05	0.19	9	1.85	0.57
55	BG	2983	20	0.70	0.11	11	0.91	0.09	9	0.78	0.15	5	6.08	0.26	5	1.57	0.40
56	BS	2983	24	0.88	0.06	12	0.58	0.15	6	0.50	0.22	6	5.95	0.29	6	1.26	0.42
57	CAR	2983	23	0.43	0.11	9	0.44	0.18	4	0.75	0.25	3	4.04	1.72	3	2.58	0.31
58	CM	2983	12	0.67	0.14	6	0.50	0.22	2	1.00	0.00	4	6.03	0.40	4	2.00	1.30
59	LUD	2983	15	0.80	0.11	11	0.45	0.16	4	0.75	0.25	4	6.35	0.23	4	1.98	0.46
60	MB	2983	21	0.71	0.10	11	0.55	0.16	6	0.83	0.17	7	6.07	0.29	7	3.45	0.54
61	MC	2983	20	0.65	0.11	9	0.44	0.18	5	0.80	0.20	5	5.91	0.17	5	1.04	0.70
62	NPB	2983	12	0.75	0.13	12	0.67	0.14	7	1.00	0.00	7	5.26	0.80	7	2.11	0.65
63	PJ	2983	19	0.74	0.10	10	0.40	0.16	3	0.67	0.33	3	6.20	0.36	3	1.47	0.25
64	TI	2983	20	0.60	0.11	12	0.67	0.14	6	0.67	0.21	7	6.31	0.18	7	1.63	0.32
65	TRUM	2983	17	0.65	0.12	8	0.38	0.18	2	1.00	0.00	3	5.68	0.21	3	2.72	0.34
66	UI	2983	16	0.44	0.13	5	0.40	0.24	1	0.00	-	1	6.40	-	1	2.34	-
67	ARAB	ARAB	11	0.91	0.09	17	0.65	0.12	4	0.00	0.00	4	5.33	0.21	4	3.36	0.92
68	BER	ARAB	30	0.77	0.08	12	0.67	0.14	6	1.00	0.00	8	5.60	0.30	8	3.05	0.72
69	BG	ARAB	30	0.93	0.05	12	0.58	0.15	7	0.86	0.14	8	5.85	0.15	8	3.36	0.62
70	BS	ARAB	33	0.82	0.07	12	0.67	0.14	8	0.88	0.13	7	5.68	0.23	7	4.61	0.72
71	CAR	ARAB	24	0.71	0.09	11	0.73	0.14	7	1.00	0.00	9	5.70	0.18	9	2.51	0.56
72	CM	ARAB	11	0.73	0.14	6	0.83	0.17	5	1.00	0.00	5	4.88	0.35	5	4.79	0.57

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
73	LUD	ARAB	19	0.79	0.10	12	0.67	0.14	8	1.00	0.00	6	5.41	0.36	6	5.39	0.42
74	MB	ARAB	23	0.70	0.10	11	0.73	0.14	6	0.83	0.17	7	4.94	0.15	7	4.96	0.52
75	MC	ARAB	24	0.75	0.09	12	0.67	0.14	7	0.86	0.14	9	5.85	0.15	9	3.37	0.38
76	NPB	ARAB	22	0.91	0.06	12	0.58	0.15	7	0.86	0.14	6	5.98	0.10	6	3.13	0.28
77	PJ	ARAB	21	0.71	0.10	12	0.33	0.14	4	1.00	0.00	5	5.69	0.20	5	4.10	1.05
78	TI	ARAB	27	0.89	0.06	12	0.67	0.14	7	0.86	0.14	7	5.19	0.26	7	4.78	0.46
79	TRUM	ARAB	24	0.50	0.10	10	0.50	0.17	5	0.80	0.20	4	5.69	0.13	4	3.98	0.41
80	UI	ARAB	19	0.63	0.11	9	0.56	0.18	4	1.00	0.00	6	5.33	0.27	6	5.40	0.35
81	BER	BER	5	1.00	0.00	15	0.73	0.12	2	0.50	0.50	1	5.48	-	1	0.40	-
82	BG	BER	33	0.97	0.03	12	0.50	0.15	8	0.75	0.16	8	5.41	0.17	8	3.14	0.46
83	BS	BER	35	0.97	0.03	10	0.60	0.16	5	1.00	0.00	7	5.02	0.15	7	4.82	0.69
84	CAR	BER	29	0.76	0.08	12	0.58	0.15	7	0.86	0.14	7	5.60	0.11	7	3.96	0.52
85	CM	BER	26	0.88	0.04	12	0.75	0.13	9	0.78	0.15	10	4.53	0.20	10	4.98	0.30
86	LUD	BER	18	0.94	0.06	11	0.36	0.15	4	0.75	0.25	3	4.35	0.26	3	5.07	0.31
87	MB	BER	20	0.95	0.05	12	0.67	0.14	5	1.00	0.00	8	4.48	0.12	8	4.74	0.52
88	MC	BER	21	0.81	0.09	12	0.67	0.14	8	0.88	0.13	8	5.61	0.14	8	3.53	0.21
89	NPB	BER	19	1.00	0.00	12	0.83	0.11	10	0.90	0.10	9	5.36	0.20	9	3.71	0.32
90	PJ	BER	22	0.82	0.08	12	0.67	0.14	8	0.88	0.13	7	4.33	0.25	7	4.34	0.54
91	TI	BER	27	0.96	0.04	12	0.75	0.13	10	1.00	0.00	9	4.77	0.26	9	4.83	0.23
92	TRUM	BER	19	0.89	0.07	12	0.58	0.15	6	1.00	0.00	8	5.93	0.18	8	1.98	0.46
93	UI	BER	31	0.81	0.07	12	0.92	0.08	10	0.90	0.10	7	4.80	0.21	7	4.88	0.65
94	BG	BG	12	1.00	0.00	20	0.60	0.11	6	0.83	0.17	7	4.91	0.36	7	5.04	0.35
95	BS	BG	35	1.00	0.00	12	0.67	0.14	8	1.00	0.00	8	4.94	0.25	8	4.25	0.59
96	CAR	BG	31	0.84	0.07	11	0.64	0.15	7	0.57	0.20	5	6.06	0.19	5	1.98	0.45
97	CM	BG	19	0.95	0.05	12	0.75	0.13	10	0.70	0.15	6	4.75	0.40	6	5.06	0.33
98	LUD	BG	29	0.93	0.05	12	0.25	0.13	4	1.00	0.00	4	4.63	0.55	4	4.92	0.68

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
99	MB	BG	25	0.96	0.04	12	0.67	0.14	9	0.89	0.11	8	5.27	0.37	8	3.81	0.48
100	MC	BG	26	0.96	0.04	12	0.83	0.11	11	0.91	0.09	10	5.61	0.13	10	3.16	0.34
101	NPB	BG	28	0.93	0.05	12	0.75	0.13	9	0.89	0.11	8	5.51	0.17	8	3.70	0.34
102	PJ	BG	26	1.00	0.00	12	0.92	0.08	11	1.00	0.00	12	4.95	0.26	12	4.31	0.34
103	TI	BG	32	0.97	0.03	12	0.83	0.11	9	1.00	0.00	7	4.82	0.26	7	4.68	0.45
104	TRUM	BG	32	0.84	0.07	11	0.55	0.16	7	1.00	0.00	6	6.17	0.18	6	1.30	0.37
105	UI	BG	31	0.97	0.03	12	0.92	0.08	11	0.73	0.14	10	4.99	0.25	10	4.68	0.51
106	BS	BS	16	1.00	0.00	23	0.67	0.10	8	1.00	0.00	11	5.23	0.08	11	4.14	0.44
107	CAR	BS	29	0.90	0.06	13	0.92	0.08	12	0.75	0.13	10	5.76	0.12	10	3.82	0.55
108	CM	BS	24	0.92	0.06	11	0.64	0.15	7	0.86	0.14	6	4.34	0.14	6	5.40	0.24
109	LUD	BS	28	0.96	0.04	11	0.45	0.16	6	0.67	0.21	7	4.27	0.15	7	4.26	0.76
110	MB	BS	28	0.86	0.07	12	0.67	0.15	8	0.75	0.16	8	4.93	0.27	8	4.91	0.68
111	MC	BS	24	0.92	0.06	12	0.58	0.13	10	0.50	0.17	9	5.12	0.21	9	3.86	0.57
112	NPB	BS	29	1.00	0.00	12	0.75	0.15	8	1.00	0.00	6	4.58	0.20	6	5.56	0.45
113	PJ	BS	26	0.96	0.04	12	0.58	0.15	12	0.92	0.08	10	4.26	0.11	10	5.03	0.26
114	TI	BS	23	0.96	0.04	12	1.00	0.00	7	0.86	0.14	6	4.25	0.26	6	5.32	0.22
115	TRUM	BS	33	0.79	0.07	11	0.58	0.15	8	0.75	0.16	8	4.61	0.33	8	3.94	0.42
116	UI	BS	26	0.85	0.07	12	0.64	0.13	9	0.89	0.11	9	4.46	0.18	9	4.70	0.80
117	CAR	CAR	10	0.90	0.10	18	0.78	0.10	5	0.80	0.20	14	4.36	0.08	14	5.32	0.26
118	CM	CAR	25	0.64	0.10	12	0.75	0.13	9	0.89	0.11	7	5.30	0.23	7	4.35	0.78
119	LUD	CAR	22	0.64	0.10	12	0.58	0.15	7	0.86	0.14	7	5.42	0.25	7	4.68	0.55
120	MB	CAR	24	0.63	0.10	12	0.50	0.15	6	1.00	0.00	6	5.17	0.54	6	4.36	0.22
121	MC	CAR	22	0.64	0.10	9	0.78	0.15	6	0.83	0.17	5	5.81	0.05	5	1.67	0.37
122	NPB	CAR	23	0.78	0.09	12	0.67	0.14	8	0.63	0.18	8	5.87	0.16	8	2.29	0.40
123	PJ	CAR	28	0.61	0.09	11	0.73	0.14	9	0.89	0.11	8	5.18	0.43	8	4.31	0.64
124	TI	CAR	31	0.77	0.08	12	0.75	0.13	8	0.63	0.18	7	5.42	0.35	7	4.61	0.48

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
125	TRUM	CAR	25	0.72	0.09	11	0.45	0.16	5	1.00	0.00	6	5.31	0.30	6	3.15	0.60
126	UI	CAR	29	0.76	0.08	10	0.70	0.15	7	0.71	0.18	6	5.10	0.18	6	5.08	0.16
127	CM	CM	3	1.00	0.00	12	0.58	0.15	2	0.50	0.50	8	5.41	0.17	8	1.62	0.36
128	LUD	CM	16	0.88	0.09	12	0.17	0.11	4	0.75	0.25	2	3.48	0.23	2	4.05	1.45
129	MB	CM	14	0.93	0.07	12	0.58	0.15	8	0.63	0.18	6	3.16	0.22	6	4.68	0.29
130	MC	CM	20	0.60	0.11	9	0.44	0.18	4	0.75	0.25	4	4.51	0.49	4	4.64	0.31
131	NPB	CM	17	1.00	0.00	12	0.67	0.14	7	0.86	0.14	6	4.91	0.43	6	4.86	0.47
132	PJ	CM	18	0.94	0.06	11	0.27	0.14	3	0.33	0.33	3	2.83	0.39	3	4.47	0.66
133	TI	CM	21	0.76	0.09	12	0.67	0.14	9	0.89	0.11	8	3.92	0.15	8	4.79	0.32
134	TRUM	CM	22	0.86	0.07	12	0.25	0.13	4	0.75	0.25	3	4.69	0.72	3	3.85	0.95
135	UI	CM	20	0.85	0.07	12	0.67	0.14	8	0.88	0.13	7	4.19	0.22	7	4.98	0.46
136	LUD	LUD	7	1.00	0.00	18	0.83	0.09	5	0.80	0.20	1	3.10	-	1	5.11	-
137	MB	LUD	22	0.95	0.05	12	0.33	0.14	5	0.40	0.24	4	3.61	0.07	4	4.49	0.56
138	MC	LUD	17	0.71	0.11	9	0.56	0.18	6	0.50	0.22	5	4.64	0.23	5	4.73	0.92
139	NPB	LUD	20	1.00	0.00	11	0.45	0.16	6	0.83	0.17	3	5.04	0.30	3	4.74	0.94
140	PJ	LUD	17	0.88	0.08	12	0.33	0.14	5	0.40	0.24	3	3.78	0.07	3	5.22	0.21
141	TI	LUD	28	0.93	0.05	12	0.58	0.15	7	0.57	0.20	5	3.82	0.19	5	5.47	0.27
142	TRUM	LUD	21	0.86	0.08	11	0.45	0.16	5	0.40	0.24	5	5.21	0.26	5	4.10	0.37
143	UI	LUD	22	0.68	0.10	6	0.67	0.21	5	0.60	0.24	4	4.68	0.08	4	6.07	0.12
144	MB	MB	5	1.00	0.00	15	0.60	0.13	-	-	-	4	3.15	0.18	4	4.78	0.44
145	MC	MB	15	0.67	0.13	9	0.67	0.17	6	1.00	0.00	6	4.63	0.45	6	3.94	0.59
146	NPB	MB	18	1.00	0.00	12	0.75	0.13	10	0.90	0.10	9	4.86	0.24	9	5.10	0.26
147	PJ	MB	17	0.88	0.08	12	0.42	0.15	6	0.67	0.21	5	3.43	0.27	5	4.81	0.54
148	TI	MB	18	0.89	0.08	12	0.50	0.15	7	1.00	0.00	6	3.64	0.10	6	5.12	0.21
149	TRUM	MB	19	0.79	0.10	12	0.42	0.15	7	0.71	0.18	5	5.24	0.48	5	4.37	0.61
150	UI	MB	19	0.84	0.09	12	0.67	0.14	8	0.88	0.13	7	4.31	0.11	7	5.57	0.19

Cross Pair	Parent A	Parent B	Cross Success			Germination			Flowered			Flower Number			Fruit Set		
			n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
151	MC	MC	10	0.90	0.10	12	0.75	0.13	6	0.50	0.22	2	3.49	0.15	2	4.63	0.07
152	NPB	MC	19	0.68	0.11	12	0.67	0.14	8	0.75	0.16	8	5.26	0.15	8	2.49	0.43
153	PJ	MC	23	0.65	0.10	6	0.67	0.21	5	0.60	0.24	4	5.68	0.45	4	4.27	1.31
154	TI	MC	23	0.65	0.10	12	0.75	0.13	9	1.00	0.00	8	4.29	0.13	8	4.48	0.75
155	TRUM	MC	27	0.59	0.10	10	0.60	0.16	6	1.00	0.00	5	6.06	0.16	5	1.26	0.46
156	UI	MC	21	0.81	0.09	11	0.64	0.15	7	0.71	0.18	6	4.98	0.28	6	5.14	0.27
157	NPB	NPB	5	1.00	0.00	15	0.73	0.12	1	1.00	-	1	6.01	-	1	2.70	-
158	PJ	NPB	12	0.92	0.08	9	0.44	0.18	5	1.00	0.00	4	4.57	0.31	4	5.23	0.08
159	TI	NPB	18	0.89	0.08	11	0.64	0.15	7	1.00	0.00	7	4.77	0.26	7	4.93	0.55
160	TRUM	NPB	20	0.85	0.08	12	0.33	0.14	5	1.00	0.00	4	6.04	0.21	4	2.28	0.47
161	UI	NPB	19	0.95	0.05	12	0.50	0.15	5	0.80	0.20	5	4.99	0.32	5	5.05	0.48
162	PJ	PJ	7	1.00	0.00	15	0.60	0.13	-	-	-	5	5.23	0.09	5	3.99	0.60
163	TI	PJ	21	0.86	0.08	12	0.58	0.15	7	0.86	0.14	6	3.29	0.17	6	4.54	0.30
164	TRUM	PJ	19	0.68	0.11	11	0.36	0.15	6	0.67	0.21	3	6.15	0.42	3	2.49	1.63
165	UI	PJ	23	0.91	0.06	11	0.64	0.15	7	0.71	0.18	6	4.36	0.17	6	5.52	0.20
166	TI	TI	4	1.00	0.00	15	0.80	0.11	1	1.00	-	2	3.61	0.01	2	4.42	0.17
167	TRUM	TI	20	0.85	0.08	9	0.56	0.18	5	1.00	0.00	6	4.29	0.92	6	3.78	0.37
168	UI	TI	23	0.87	0.07	12	0.67	0.14	9	0.78	0.15	8	4.24	0.07	8	5.34	0.42
169	TRUM	TRUM	7	1.00	0.00	16	0.69	0.12	1	0.00	-	7	3.30	0.16	7	4.42	0.70
170	UI	TRUM	24	0.88	0.07	12	0.25	0.13	3	0.67	0.33	4	5.83	0.31	4	2.47	0.58
171	UI	UI	9	0.89	0.11	15	0.80	0.11	-	-	-	4	4.24	0.29	4	5.27	0.40

Table 18. Functional trait values for parent taxa (see Table 15 for species codes). Gluco. PC1 is the first axis of a principle component analysis of glucosinolate profile data from Rodman (1974) (see Material and Methods). Leaf lobedness was measured as perimeter (cm) / area (cm^2). n = sample size of leaves (2 per 5 – 6 genotypes), SE = standard error.

		Leaf Lobedness		
Species	**Gluco. PC1**	**n**	**mean**	**SE**
1396	-1.44	10	0.06	0.01
1944	NA	12	0.19	0.06
2055	NA	12	0.25	0.05
2983	NA	10	0.12	0.02
ARAB	NA	NA	NA	NA
BER	-44.38	12	0.06	0.01
BG	51.98	12	0.06	0.01
BS	48.77	12	0.07	0.01
CAR	-3.30	12	0.12	0.02
CM	-54.54	10	0.06	0.02
LUD	-63.04	10	0.08	0.02
MB	-63.04	10	0.07	0.02
MC	56.94	11	0.06	0.01
NPB	53.30	12	0.05	0.01
PJ	-54.54	12	0.05	0.02
Ti	60.15	12	0.06	0.02
TRUM	-62.13	10	0.07	0.03
UI	53.30	12	0.04	0.01

Table 19. Geographic and climatic values for parent taxa (see Table 15 for species codes). Values represent means from geospatial and climatic data sampled across the range of species (the full dataset across the range was used to compute principle components, see Material and Methods). Geographic distance was calculated using latitude and longitude values below. n = sample size (number of geospatial coordinates), SE = standard error.

		Altitude		Longitude		Latitude		Annual Mean Temp.		Temp. Warmest Qrt.		Temp. Coldest Qrt.		Annual Precip.		Precip. Wettest Mo.		Precip. Driest Mo.		Precip. Seasonality		Precip. Wettest Qrt.	
taxa	n	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
1396	**255**	1.00	-	-1.79	0.36	53.24	0.32	93.38	1.34	149.83	1.31	40.40	1.58	1013.52	19.62	117.71	2.66	53.54	1.02	24.33	0.56	334.67	7.55
1944	9	1112.33	89.68	-5.02	0.61	31.28	0.28	182.78	8.34	281.22	10.25	90.56	6.89	151.78	25.66	25.89	4.32	2.00	0.58	58.44	5.78	67.56	10.84
2055	100	157.92	16.84	24.98	1.45	37.45	0.86	175.51	4.43	242.58	4.32	107.16	4.44	527.81	16.74	110.77	3.99	7.14	1.56	85.03	3.39	287.91	9.86
2983	4	485.00	151.48	-10.07	0.13	29.05	0.13	181.00	7.05	226.50	2.87	133.00	9.64	142.25	18.19	29.50	1.50	1.00	0.00	74.50	2.53	73.25	8.04
ARAB	16	453.44	139.22	45.72	0.84	27.03	0.90	240.62	5.69	329.06	7.51	141.25	5.59	114.75	12.56	27.00	2.72	0.06	0.06	93.06	2.26	63.69	6.48
BER	5	1.00	-	-64.78	0.03	32.29	0.01	215.00	0.32	264.00	0.32	171.80	0.37	1473.60	3.22	161.20	1.07	97.60	0.51	12.80	0.37	433.40	0.75
BG	3	1.00	-	-81.76	0.24	24.14	0.49	250.00	1.15	281.00	3.61	213.33	4.10	1113.33	79.80	173.67	7.26	42.33	2.03	49.33	1.86	434.33	17.70
BS	12	1.00	-	-89.51	0.69	29.97	0.09	200.42	0.61	273.25	0.46	119.00	1.23	1536.25	14.62	178.58	4.50	85.50	1.54	21.75	1.28	504.17	10.51
CAR	79	1.00	-	-119.21	3.11	40.30	0.57	123.35	3.18	166.89	2.39	82.78	4.90	1141.00	83.78	201.37	12.97	11.68	1.57	77.46	1.86	558.72	37.86
CM	6	1.00	-	-75.18	0.46	39.40	0.43	127.67	3.69	234.17	2.91	18.33	4.65	1097.50	20.14	110.00	1.63	76.50	1.43	11.00	0.52	304.83	3.46
LUD	21	1.00	-	-80.22	0.55	42.38	0.20	91.29	1.88	207.86	1.90	-32.33	2.14	911.90	17.22	94.33	1.59	52.10	1.68	16.90	0.69	268.00	4.77
MB	17	1.00	-	-87.48	0.19	43.59	0.27	77.71	2.68	198.00	2.63	-52.59	3.45	824.71	13.24	95.65	1.21	33.12	1.27	28.35	1.13	272.65	3.55
MC	3	1.00	-	-82.75	0.05	27.66	0.17	223.67	0.33	275.67	0.88	163.00	1.53	1280.33	10.87	221.00	4.93	39.33	3.84	56.67	2.40	598.67	12.20
NPB	6	1.00	-	-80.05	0.19	25.97	0.26	242.33	2.04	279.67	1.45	198.67	2.93	1360.83	67.47	199.00	5.25	51.50	3.21	46.83	2.30	501.50	17.69
PJ	26	1.00	-	-68.38	1.01	43.44	0.48	84.62	3.96	193.35	3.93	-26.38	4.28	1154.96	15.74	120.08	2.37	79.00	1.00	12.50	0.44	334.31	6.18
Ti	14	1.00	-	-78.40	0.62	33.78	0.48	176.50	3.41	259.14	1.88	88.29	4.74	1329.71	15.93	172.50	3.57	69.93	2.97	28.71	1.78	476.57	8.73
TRUM	53	1.00	-	-21.43	0.33	64.98	0.13	37.85	1.65	92.66	1.38	-6.75	1.93	968.13	24.76	110.57	3.02	54.06	1.65	21.64	0.26	297.94	7.45
UI	14	1.00	-	-68.12	2.24	15.03	0.93	267.00	1.07	276.07	0.72	254.36	2.13	1537.86	128.52	217.00	11.78	45.50	6.03	49.64	3.73	576.57	35.71

		Precip. Driest Qtr.		Precip. Warmest Qtr.		Precip. Coldest Qtr.		Diurnal Range		Isothermality		Temp. Seasonality		Max. Temp. Warmest Qtr.		Min. Temp. Warmest Qtr.		Annual Temp. Range		Temp. Wettest Qtr.		Temp. Driest Qtr.	
taxa	n	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
1396	**255**	175.52	3.11	221.31	4.56	279.78	6.01	63.76	0.70	34.50	0.34	4341.49	45.75	193.13	1.61	10.01	1.51	183.12	1.45	81.85	1.95	97.69	2.83
1944	9	10.22	1.75	12.33	2.09	46.56	13.03	156.89	4.51	40.67	1.12	7383.56	193.43	388.67	9.94	7.89	6.66	380.78	6.20	153.78	14.56	229.56	24.91
2055	100	26.52	5.21	35.56	6.80	260.38	9.78	97.21	2.27	39.45	0.64	5369.55	70.46	302.19	5.01	60.71	4.01	241.48	3.34	128.38	3.35	228.23	7.30
2983	4	4.00	0.00	8.75	1.03	68.25	6.01	100.00	2.61	48.25	1.31	3577.75	261.89	280.50	1.26	74.75	10.94	205.75	10.07	142.50	9.92	223.00	3.34
ARAB	16	0.69	0.62	2.81	2.68	45.75	5.22	134.31	4.93	40.12	1.49	7396.56	285.61	407.69	8.36	74.69	6.32	333.00	8.99	171.06	10.96	324.44	10.86
BER	5	321.80	1.66	390.40	0.24	354.00	2.21	55.40	0.68	35.00	0.32	3621.40	6.55	299.40	0.24	143.60	0.40	155.80	0.37	255.00	0.32	190.20	0.20
BG	3	138.00	7.64	404.67	15.32	152.00	8.89	73.33	8.25	48.67	5.24	2664.67	275.68	320.00	1.00	172.00	3.21	148.00	3.21	274.00	2.65	229.00	2.89
BS	12	302.75	5.20	482.58	10.75	366.67	7.74	97.42	1.96	36.58	0.70	6056.50	56.57	322.00	0.70	58.75	0.97	263.25	1.17	270.25	0.35	164.00	0.92
CAR	79	55.95	6.62	68.49	7.66	525.42	35.43	99.38	2.57	52.19	0.72	3325.82	166.75	227.16	3.21	35.59	5.03	191.57	6.10	90.47	3.50	157.70	4.99
CM	6	248.00	3.86	299.50	3.32	250.00	3.73	101.83	3.53	29.67	0.95	8386.50	105.51	297.83	4.02	-38.00	3.28	335.83	3.45	229.50	3.22	49.00	23.18
LUD	21	176.76	4.52	256.10	5.42	186.95	6.05	100.52	0.97	27.14	0.24	9333.52	50.39	276.86	1.97	-86.86	2.63	363.71	2.04	162.62	13.40	-19.67	2.32
MB	17	126.00	4.83	262.76	5.33	128.24	5.63	101.29	2.36	26.06	0.39	9718.18	95.41	268.18	2.54	-112.71	4.56	380.88	5.02	188.18	5.40	-51.12	3.68
MC	3	180.67	4.70	574.67	24.44	203.00	4.58	102.00	2.08	45.33	0.88	4481.00	101.17	325.00	0.58	102.67	1.20	222.33	1.76	275.67	0.88	174.33	1.76
NPB	6	174.17	12.10	479.67	20.06	174.83	11.67	83.50	5.25	47.50	1.15	3176.17	67.72	322.67	1.73	149.00	6.02	173.67	7.16	271.50	1.52	201.67	4.90
PJ	26	250.19	3.36	256.12	2.90	301.54	4.37	99.23	2.05	28.12	0.32	8546.85	93.71	260.46	3.83	-85.15	4.78	345.62	4.57	26.92	6.01	171.46	7.28
Ti	14	244.86	8.61	461.71	8.49	283.14	6.98	109.29	2.33	36.86	0.73	6667.57	111.55	315.21	2.12	22.93	4.37	292.29	3.14	256.64	1.94	131.36	4.23
TRUM	53	170.75	4.84	190.30	4.91	281.00	7.60	50.49	0.75	31.17	0.25	4002.51	40.03	125.89	1.18	-33.45	2.31	159.34	1.70	14.08	2.42	75.45	2.08
UI	14	155.43	19.58	497.14	50.53	186.29	26.57	77.00	1.38	74.29	1.89	870.36	94.45	316.36	1.17	212.57	2.18	103.79	2.95	272.79	0.65	256.64	2.49

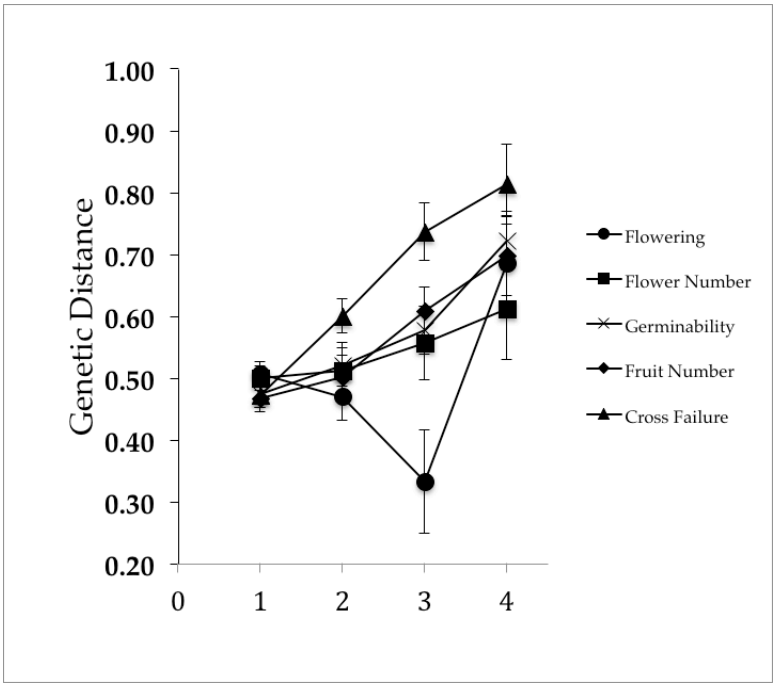


Figure 11. Comparison of evolutionary rates of different components of reproductive isolation. Rates were assessed by comparing mean genetic distance at different levels (1: < 0.25, 2: < 0.50, 3: < 0.75, 4: < 1) of reproductive isolation for each component of reproductive isolation (see key).

To test if post-zygotic reproductive isolation increased with time, we tested if each stage of RI was correlated with genetic distance. For both non-parametric and parametric tests, we found cross failure, seed germinability, and fruit set to be significantly correlated with genetic distance (Table 22). Cumulative measures of post-zygotic reproductive isolation for fecundity, total survival and total fitness were also significantly correlated with genetic distance (Table 22). Analysis with phylogenetically corrected data (PICs) found similar associations, with the exception that the correlations with fruit set and total fitness were non-significant (Table 22). Overall, post-zygotic reproductive

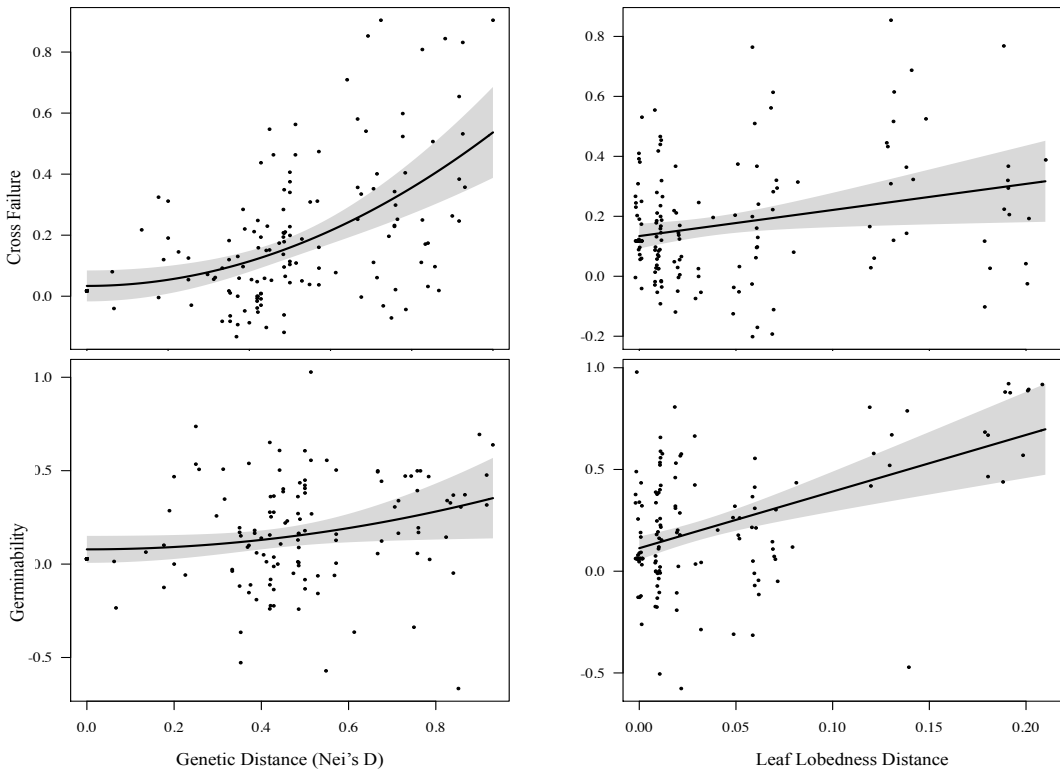


Figure 12. Correlation of reproductive isolation (RI) with genetic distance (left two panels) and ecological distance (right two panels). Measures of RI include cross failure and F_1 germinability. Ecological distance is represented by distance between parental taxa in the functional trait of leaf lobedness. Regression slopes are black lines. Grey shadow indicates 95% confidence interval of regression slopes. Regression slopes are based on multivariate analysis including both genetic distance and leaf lobedness as independent variables. Genetic distance was included as a non-linear variable based on independent analysis of its relationship to RI (Table 25).

isolation tended to increase with genetic distance, but it seems to be strongest in early stages of F_1 survival (Table 22).

Associations between ecological distance and post-zygotic RI. Some measures of ecological distance were positively correlated with genetic distance (Table 23). Geographic distance, and differences in annual precipitation increased with

genetic distance in phylogenetically corrected analyses, and niche overlap decreased with genetic distance (Table 23). Leaf lobedness, and glucosinolate profiles also increased with genetic distance in uncorrected analyses (Table 23). Latitudinal distance and temperature seasonality had no relationship with genetic distance (Table 23).

There were few associations between measures of RI and measures of ecological distance (Table 24). Cross failure and seed germinability were both significantly correlated with leaf lobedness (Figure 12, Table 24). Analysis with PICs found that the association of seed germinability and cross failure with leaf lobedness was in the same direction and had a similar magnitude, but was non-significant (Table 24).

Test of non-linear accumulation of RI. To test if the accumulation of post-zygotic reproductive isolation was nonlinear, we compared both linear and nonlinear models based on comparison of AIC scores. Earlier stages of post-zygotic reproductive isolation were nonlinear, while later stages were linear (Figure 12, Table 25). There was a significant nonlinear accumulation of cross failure and seed germinability (Figure 12, Table 25), while for fruit set and cumulative F1-fecundity accumulation was linear (Table 25). This was reflected in the cumulative total reproductive isolation: the best-fit model for total isolation that included fecundity ($RI_{\text{Total Fitness}}$) was linear (although the snowball model was also

significant), while the best-fit model for total isolation that did not include fecundity ($RI_{Total\ Survival}$) was nonlinear (Table 25). Analysis with PICs found slightly different results. The best-fit model for the accumulation of cross failure remained nonlinear, while the model of the accumulation of seed germinability became linear (Table 25). The accumulation of reproductive isolation that included measures of fecundity was non-significant (Table 25). The best-fit model for $RI_{Total\ Survival}$ was non-linear, but also non-significant, while the best-fit significant model was linear (Table 25).

Table 20. Estimates of reproductive isolation. 'F_1 Survival' is the cumulative effect of seed germinability and survival to flower. 'F_1 Fecundity' is the cumulative effect of flower number and fruit set. 'Total Survival' includes Cross Failure and F_1 Survival. 'Total Fitness' includes all five stages.

Stage	*N (pairs)*	Mean RI	SE	Range
Cross Failure	153	0.27	0.02	-0.02 – 1.00
Germination	139	0.26	0.03	-0.54 – 1.00
Flowering	126	0.21	0.02	0.00 – 1.00
Flower Number	127	0.00	0.05	-1.50 – 1.00
Fruit Set	127	0.14	0.05	-3.18 – 0.99
F1 Survival	126	0.35	0.03	-0.53 – 1.00
F1 Fecundity	127	0.15	0.06	-1.89 – 1.00
Total Survival	126	0.40	0.03	-1.60 – 1.00
Total Fitness	125	0.53	0.04	-0.81 – 1.00

Table 21. Comparison of evolutionary rates across different stages of reproductive isolation. Differences in mean genetic distance across stages of RI were tested for at different levels of RI (< 0.25, < 0.50, < 0.75, < 1) using both parametric (ANOVA) and non-parametric (Mann-Whitney) statistics. ANOVA comparisons were adjusted using a Tukey HSD correction for multiple comparisons.

			ANOVA					Mann-Whitney	
RI Level	Comparison		β	SE	df	t	P	U	P
< 0.25	Flowering	Flower Number	0.01	0.03	411	0.29	0.9984	3793.5	0.4669
< 0.25	Flowering	Germination	0.03	0.03	411	1.22	0.7380	3115.5	0.1784
< 0.25	Flowering	Fruit Set	0.04	0.03	411	1.44	0.6033	2776.5	0.0760
< 0.25	Flowering	Cross Failure	0.03	0.03	411	1.29	0.6946	3510.0	0.1557
< 0.25	Flower Number	Germination	0.03	0.03	411	0.93	0.8851	3203.5	0.5474
< 0.25	Flower Number	Fruit Set	0.03	0.03	411	1.15	0.7804	2835.5	0.2543
< 0.25	Flower Number	Cross Failure	0.03	0.03	411	0.99	0.8592	3585.0	0.4688
< 0.25	Germination	Fruit Set	0.01	0.03	411	0.23	0.9994	2619.0	0.5620
< 0.25	Germination	Cross Failure	0.00	0.03	411	0.03	1.0000	3310.0	0.8977
< 0.25	Fruit Set	Cross Failure	-0.01	0.03	411	-0.21	0.9996	3260.0	0.6588
< 0.50	Flowering	Flower Number	-0.04	0.05	146	-0.80	0.9287	249.5	0.2283
< 0.50	Flowering	Germination	-0.05	0.05	146	-1.00	0.8528	257.0	0.1489
< 0.50	Flowering	Fruit Set	-0.03	0.05	146	-0.62	0.9715	272.5	0.2470
< 0.50	Flowering	Cross Failure	**-0.13**	**0.05**	**146**	**-2.79**	**0.0464**	**304.0**	**0.0030**
< 0.50	Flower Number	Germination	-0.01	0.05	146	-0.19	0.9997	257.0	0.1489
< 0.50	Flower Number	Fruit Set	0.01	0.05	146	0.20	0.9996	272.5	0.2470
< 0.50	Flower Number	Cross Failure	-0.09	0.05	146	-1.94	0.3019	462.5	0.1451
< 0.50	Germination	Fruit Set	0.02	0.05	146	0.40	0.9946	400.5	0.8955
< 0.50	Germination	Cross Failure	-0.08	0.04	146	-1.77	0.3955	490.0	0.1130
< 0.50	Fruit Set	Cross Failure	-0.10	0.04	146	-2.21	0.1808	**803.5**	**0.0495**
< 0.75	Flowering	Flower Number	-0.23	0.10	58	-2.21	0.1910	1.0	0.0136
< 0.75	Flowering	Germination	*-0.24*	*0.09*	*58*	*-2.68*	*0.0701*	**12.5**	**0.0423**
< 0.75	Flowering	Fruit Set	**-0.28**	**0.09**	**58**	**-3.01**	**0.0305**	**4.0**	**0.0065**
< 0.75	Flowering	Cross Failure	**-0.40**	**0.10**	**58**	**-4.24**	**0.0007**	**0.5**	**0.0045**
< 0.75	Flower Number	Germination	-0.02	0.07	58	-0.28	0.9986	70.5	0.7903
< 0.75	Flower Number	Fruit Set	-0.05	0.07	58	-0.72	0.9513	65.5	0.5944
< 0.75	Flower Number	Cross Failure	-0.18	0.07	58	-2.39	0.1326	**16.0**	**0.0100**
< 0.75	Germination	Fruit Set	-0.03	0.05	58	-0.57	0.9795	198.0	0.6192
< 0.75	Germination	Cross Failure	*-0.16*	*0.06*	*58*	*-2.65*	*0.0741*	**64.5**	**0.0246**
< 0.75	Fruit Set	Cross Failure	-0.13	0.06	58	-2.14	0.2166	**66.5**	**0.0299**
< 1	Flowering	Flower Number	0.07	0.11	37	0.68	0.9605	18.0	0.6623
< 1	Flowering	Germination	-0.04	0.09	37	-0.41	0.9939	36.5	0.5329
< 1	Flowering	Fruit Set	-0.01	0.10	37	-0.11	1.0000	24.5	1.0000
< 1	Flowering	Cross Failure	-0.13	0.10	37	-1.29	0.6992	14.5	0.2448
< 1	Flower Number	Germination	-0.11	0.09	37	-1.17	0.7657	32.0	0.6620

RI Level	Comparison		β	SE	df	t	P	U	P
< 1	Flower Number	Fruit Set	-0.09	0.10	37	-0.82	0.9222	16.0	0.6216
< 1	Flower Number	Cross Failure	-0.20	0.10	37	-1.94	0.3155	11.0	0.2121
< 1	Germination	Fruit Set	0.03	0.08	37	0.31	0.9978	69.0	0.5828
< 1	Germination	Cross Failure	-0.09	0.08	37	-1.14	0.7844	42.5	0.2723
< 1	Fruit Set	Cross Failure	-0.12	0.09	37	-1.27	0.7084	20.0	0.2345

Table 22. Correlations between reproductive isolation and genetic distance. Analyses used both uncorrected and phylogenetically corrected data, as well as, parametric (linear regression) and non-parametric (Kendall's τ) statistics. For analyzes with uncorrected data, significance was adjusted by the number of cross pairs a taxon could be replicated across (18) using a Bonferonni correction (α = 0.0028). Significant correlations ($P_{adjusted}$ = 0.05) are in bold. Phylogenetically corrected data represent median values from analysis across 100 maximum-likelihood bootstrap phylogenies.

		Uncorrected Analysis						**PIC Corrected Analysis**					
		Non-parametric			**Parametric**			**Non-parametric**			**Parametric**		
	N	**τ**	**Z**	**P**	**β**	**P**	**R^2**	**τ**	**Z**	**P**	**β**	**P**	**R^2**
Cross Failure	171	**0.51**	**9.72**	**0.0000**	**0.60**	**0.0000**	**0.41**	**0.63**	**108.00**	**0.0003**	**1.10**	**0.0001**	**0.65**
Germination	157	**0.32**	**5.87**	**0.0000**	**0.61**	**0.0000**	**0.19**	*0.33*	*79.00*	*0.0788*	**0.98**	**0.0119**	**0.33**
Flowering	136	-0.02	-0.35	0.7296	0.00	0.9730	-0.01	0.08	1.41	0.5896	0.06	0.6585	-0.06
Flower Number	137	0.16	2.82	0.0049	0.44	0.0206	0.03	0.14	58.50	0.4951	0.38	0.5625	-0.05
Fruit Set	137	**0.27**	**4.67**	**0.0000**	**0.71**	**0.0015**	**0.07**	0.14	58.50	0.4951	0.01	0.7487	-0.07
F1 Survival	136	0.14	2.46	0.0139	0.27	0.0280	**0.03**	0.18	60.00	0.3795	0.50	0.1589	0.08
F1 Fecundity	137	**0.28**	**4.88**	**0.0000**	**1.25**	**0.0000**	**0.14**	0.12	59.00	0.4951	0.46	0.5712	-0.05
Total Survival	136	**0.18**	**3.00**	**0.0027**	**0.33**	**0.0078**	**0.04**	**0.41**	**71.00**	**0.0359**	**0.84**	**0.0075**	**0.40**
Total Fitness	134	**0.33**	**5.60**	**0.0000**	**0.90**	**0.0000**	**0.18**	*0.34*	*66.00*	*0.0836*	0.68	0.1147	0.12

Table 23. Correlations between genetic distance and ecological distance. Analyses used both uncorrected and phylogenetically corrected data. For analyses with uncorrected data, significance was adjusted by the number of cross pairs a taxon could be replicated across (18) using a Bonferonni correction (α = 0.0028). Significant correlations ($P_{adjusted}$ = 0.05) are in bold. Phylogenetically corrected data represent median values from analysis across 100 maximum-likelihood bootstrap phylogenies.

PIC Corrected Parametric	**Linear Model**		
Variable	**β**	**P**	**AIC**
Geographic Distance	**9040.2**	**0.0009**	**304.8**
Latitudinal Distance	3.4	0.6975	118.4
Climate PC1 (Temp. Seasonality)	24.6	0.7899	295.4
Climate PC2 (Annual Precip.)	**846.8**	**0.0073**	**233.6**
Niche Overlap	**-0.5**	**0.0366**	**-6.0**
Leaf Lobedness	4.1	0.1157	74.1
Glucosinolate Distance	182.1	0.0646	136.0

Uncorrected Parametric	**Linear Model**		
Variable	**β**	**P**	**AIC**
Geographic Distance	**8767.3**	**0.0000**	**3160.0**
Latitudinal Distance	7.6	0.0169	1291.0
Climate PC1 (Temp. Seasonality)	1867.6	0.0034	3103.8
Climate PC2 (Annual Precip.)	**1558.3**	**0.0000**	**2480.4**
Niche Overlap	**-0.9**	**0.0000**	**-23.5**
Leaf Lobedness	**0.2**	**0.0000**	**-525.0**
Glucosinolate Distance	**171.8**	**0.0000**	**1072.2**

Table 24. Correlations between reproductive isolation and ecological distance, controlling for genetic distance. The major component of climate PC1 is temperature seasonality, and for climate PC2 it is annual precipitation. Analyses used both uncorrected and phylogenetically corrected data. Estimates and p-values for each measure of ecological distance are presented from a multivariate analysis that included genetic distance as a covariate. For analyses with uncorrected data, significance was adjusted by the number of cross pairs a taxon could be replicated across + the number of independent variables (18 + 7 = 25) using a Bonferonni correction (α = 0.002). For corrected data, significance was adjusted for number of independent variables (7, α = 0.007). Significant correlations ($P_{adjusted}$ = 0.05) are in bold. Phylogenetically corrected data represent median values from analysis across 100 maximum-likelihood bootstrap phylogenies.

	Geographic Distance		Latitude		Climate PC1		Climate PC2		Niche Overlap		Leaf Lobedness		Glucosinolates	
RI	**β**	**P**	**β**	**P**	**β**	**P**	**β**	**P**	**β**	**P**	**β**	**P**	**β**	**P**
Uncorrected														
Cross Failure	0.00	0.0360	0.00	0.6336	0.00	0.2250	0.00	0.0831	0.16	0.0123	**1.30**	**0.0001**	0.00	0.0369
Germination	0.00	0.2860	0.00	0.9100	0.00	0.2060	0.00	0.3730	0.12	0.2920	**2.90**	**0.0000**	0.00	0.7425
Flowering	0.00	0.0915	0.00	0.3980	0.00	0.1320	0.00	0.3380	0.08	0.4436	0.88	0.2108	0.00	0.2400
Flower Number	0.00	0.0574	0.00	0.9724	0.00	0.0292	0.00	0.5502	0.35	0.0858	1.49	0.2950	0.00	0.6690
Fruit Set	0.00	0.8292	0.00	0.9293	0.00	0.0518	0.00	0.8525	-0.42	0.0817	-2.63	0.1129	0.00	0.4020
F1 Survival	0.00	0.2148	0.00	0.6802	0.00	0.1709	0.00	0.8684	0.02	0.9026	2.14	0.0085	0.00	0.5608
F1 Fecundity	0.00	0.5677	0.00	0.8686	0.00	0.6905	0.00	0.5763	-0.21	0.4631	-1.43	0.4801	0.00	0.8610
Total Survival	0.00	0.8005	0.00	0.2587	0.00	0.2207	0.00	0.8853	-0.02	0.8827	1.26	0.1329	0.00	0.4536
Total Fitness	0.00	0.8440	0.00	0.4670	0.00	0.1900	0.00	0.3425	-0.19	0.2823	0.10	0.9350	0.00	0.9495
PIC Corrected														
Cross Failure	0.00	0.3500	-0.01	0.3100	0.00	0.6300	0.00	0.7300	0.34	0.3000	1.00	0.3300	0.00	0.6500
Germination	0.00	0.6800	0.01	0.1600	0.00	0.5400	0.00	0.6100	-0.39	0.4300	3.26	0.0200	0.00	0.4000
Flowering	0.00	0.2500	-0.01	0.0900	0.00	0.5800	0.00	0.4400	0.23	0.5800	2.87	0.2200	0.00	0.5400
Flower Number	0.00	0.2900	0.00	0.6700	0.00	0.5300	0.00	0.6800	0.45	0.4500	-0.07	0.7900	0.00	0.6100
Fruit Set	0.00	0.1700	0.00	0.7100	0.00	0.5300	0.00	0.7400	0.05	0.7000	-2.82	0.3000	0.00	0.4900
F1 Survival	0.00	0.5400	0.01	0.5800	0.00	0.5300	0.00	0.6400	-0.41	0.4400	3.25	0.2800	0.00	0.5100
F1 Fecundity	0.00	0.6300	0.00	0.7700	0.00	0.4500	0.00	0.5300	0.59	0.3800	-1.46	0.7000	0.00	0.6500
Total Survival	0.00	0.3100	0.00	0.6700	0.00	0.5000	0.00	0.6000	-0.42	0.3300	1.15	0.5500	0.00	0.4600
Total Fitness	0.00	0.4800	0.00	0.6600	0.00	0.2700	0.00	0.5500	0.09	0.7300	1.12	0.6500	0.00	0.4900

Table 25. Comparison of linear and nonlinear models of the evolution of reproductive isolation. Three models were compared: linear model ($y = ax$), snow-ball model 1 ($y = ax^2$), and snow-ball model 2 ($y = ax + bx^2$). Best-fit was assessed by AIC score. Models with the lowest (best) AIC score are highlighted in blue. Analyzes used both uncorrected and phylogenetically corrected data. For analyses with uncorrected data, significance was adjusted by the number of cross pairs a taxon could be replicated across (18) using a Bonferonni correction ($\alpha = 0.0028$). Significant correlations ($P_{adjusted} = 0.05$) are in bold. Phylogenetically corrected data represent median values from analysis across 100 maximum-likelihood bootstrap phylogenies.

Uncorrected	Linear Model			Snow-ball Model 1			Snow-ball Model 2				
	β	P	AIC	β	P	AIC	β1	P	β2	P	AIC
Cross Failure	0.60	0.0000	-88.37	**0.65**	**0.0000**	**-94.80**	0.16	0.3455	0.49	0.0074	-93.71
Germination	0.61	0.0000	72.83	**0.70**	**0.0000**	**69.97**	0.12	0.6868	0.57	0.0855	71.81
Flowering	0.00	0.9730	19.07	0.06	0.5980	18.79	-0.51	0.0987	0.57	0.0832	17.99
Flower Number	0.44	0.0206	197.59	0.50	0.0122	196.65	-0.10	0.8590	0.61	0.3310	198.62
Fruit Set	**0.71**	**0.0015**	**240.73**	0.63	0.0078	243.80	1.40	0.0422	-0.78	0.2853	241.56
F1 Survival	0.27	0.0280	70.69	0.35	0.0080	68.46	-0.35	0.3458	0.70	0.0799	69.54
F1 Fitness	**1.25**	**0.0000**	**293.93**	1.19	0.0001	298.35	1.87	0.0268	-0.69	0.4385	295.31
Total 1	**0.90**	**0.0000**	**149.41**	0.91	0.0000	152.02	0.83	0.1100	0.07	0.8940	151.40
Total 2	0.33	0.0078	72.936	**0.38**	**0.0039**	**71.66**	-0.08	0.8414	0.46	0.2579	73.62

Corrected	Linear Model			Snow-ball Model			Snow-ball Model 2				
RI Type	β	P	AIC	β	P	AIC	β1	P	β2	P	AIC
Cross Failure	1.10	0.0001	-12.39	**1.21**	**0.0000**	**-15.45**	-0.11	0.7912	1.30	0.1399	-13.50
Germination	**0.98**	**0.0119**	**4.03**	1.03	0.0157	4.66	1.12	0.4331	-0.15	0.7520	5.82
Flowering	0.06	0.6585	-9.84	0.04	0.7298	-9.81	0.44	0.5926	-0.36	0.6170	-8.11
Flower Number	0.38	0.5625	21.65	0.71	0.3399	21.09	-2.93	0.2279	3.80	0.1587	21.47
Fruit Set	0.01	0.7487	10.28	-0.04	0.7446	10.33	0.34	0.7324	-0.41	0.7712	12.06
F1 Survival	0.50	0.1589	2.95	0.44	0.2733	4.05	1.73	0.2175	-1.41	0.3541	3.47
F1 Fitness	0.46	0.5712	26.82	0.73	0.3854	26.22	-2.37	0.4071	3.33	0.2962	27.10
Total 1	0.68	0.1147	6.18	*0.83*	*0.0788*	*5.84*	-0.30	0.7799	1.18	0.5040	7.65
Total 2	0.84	0.0075	-6.97	0.82	0.0296	-5.06	*2.03*	*0.0647*	*-1.34*	*0.2178*	*-7.19*

4.4 Discussion

Ecological divergence was associated with intrinsic post-zygotic reproductive isolation, but only at the earliest stages of hybrid inviability, and only to a limited degree. Functional trait divergence had significant associations with RI, but climatic divergence did not. Different components of intrinsic post-zygotic RI evolved at different rates and attained different total values. In particular, early stages of hybrid inviability evolved slowly at first, but accelerated with time in a non-linear or "snowball" fashion, and ultimately reached a greater magnitude. Thus, early stages of intrinsic post-zygotic RI appear to attain a greater magnitude driven in part by adaptive divergence and non-linear dynamics.

Intrinsic post-zygotic isolation was strongest in the earliest stages of hybrid development and survival. The consecutive barriers of cross failure, seed germinability, and survival to flower were up to 20 times greater than barriers at later stages of hybrid fecundity (flower number, fruit set). Similar to this result, comparisons of post-zygotic barriers in plants (Lowry et al. 2008a; Widmer et al. 2009) have found high reproductive isolation for hybrid seed formation (akin to our cross failure), but not for hybrid seed germinability or flowering. Furthermore, Lowry et al. (2008) reported relatively lower rates of reproductive isolation in hybrid fecundity (seed set) than viability, although there was a large

degree of variability across studies. These discrepancies could arise from the difficulty of using flower number as proxy for fitness when comparing hybrids of parents with varying degrees of self-compatibility. For instance, flower production maybe limited in hybrids with self-compatible, closely related species because they are able to set fruit. In contrast, flower production may not be limited in distantly related taxa that are either self-incompatible or genetically incompatible because they are unable to set fruit (and thus keep producing flowers). A preliminary study of out-crossing *Cakile* found no evidence for pollination affecting flower number, suggesting that flower number is fair measure of fecundity (see Chapter 4, Material and Methods). Nonetheless, the role of mating system in measures of hybrid fecundity requires further investigation.

The rate and pattern of the accumulation of RI with time (genetic distance) also differed between the earlier stages of hybrid survival and the later stages of hybrid fecundity. Firstly, the overall rate of evolution of cross failure was slower than those for all other measures of RI, which were similar to each other. Secondly, both early- and late-stage reproductive barriers were positively correlated with genetic distance, but earlier stages tended to have stronger, non-linear associations with genetic distance, while later stages tended to have

weaker, linear associations. Cross failure had the most robust non-linear relationship, holding up across phylogenetic corrected and uncorrected analyses.

The tendency for earlier stages of hybrid development, particularly seed development, to exhibit slower, non-linear evolutionary trends might reflect two processes. First, it could be the combined artifact of heterosis and the bounded range of probabilistic data. We found evidence of heterosis for measures of hybrid fecundity among closely related taxa, indicated by negative values of reproductive isolation. In contrast, for measures of survival that are necessarily scored between 0 and 1, such as cross failure, negative reproductive isolation values are not possible. Instead, heterosis would be indistinguishable from no reproductive isolation. This would result in apparent lower levels of reproductive isolation persisting for greater genetic distance, and, in effect, 'bending' the distribution such that it was non-linear. Secondly, the non-linearity we observe could reflect a real 'snowball' effect among earlier stages of post-zygotic reproductive isolation, driven by the evolution of genic incompatibilities. Evidence for 'snowball' patterns in plants is limited (Moyle and Nakazato 2010), but growing, although to date no such pattern has been tested for in early stages of hybrid survival. Finally, it is worth noting that a non-linear increase of the magnitude of RI with genetic distance does not necessarily indicate a non-linear increase in the number of incompatible loci, as predicted by the snowball effect

(Presgraves 2010). At this stage, we cannot distinguish between these alternative explanations for the non-linear accumulation of RI.

Geographic and ecological distance were only associated with RI at the earliest life stage, and then only weakly. Our results are therefore in contrast to previous studies that have identified positive associations between post-zygotic intrinsic reproductive isolation and geographic and ecological distance (Funk et al. 2006; Jewell et al. 2012). While associations between geographic distance and RI are not uncommon in plants (Jewell et al. 2012), associations with ecological divergence are less so and have only been observed in large-scale comparative studies across multiple genera or, more recently, in specific cases of recently divergent populations (Funk et al. 2006). At the intermediate scale of our study—between populations and genera—we found limited evidence of an ecological signature in the evolution of reproductive isolation. We focused on the most obvious measure of ecological divergence in the clade, climate; however, our metrics of ecological divergence may nonetheless not have captured the primary vector of selection acting on these divergent populations. In addition, we did not examine other relevant measures of hybrid fertility that can exhibit intrinsic RI (e.g., pollen viability, ovule number). In Funk et al.*'s* (2006) examination of post-zygotic reproductive isolation and ecological divergence, their metric of reproductive isolation was hybrid pollen viability, which they found to be

positively correlated with habitat divergence. It is possible that these measures of hybrid fertility might exhibit stronger associations with ecological divergence in this system, although we found no such association in another study that compared ecologically similar versus divergent sister taxa (see Chapter 5).

Nonetheless, this study found that geographic distance was marginally correlated with cross failure, but this relationship did not persist with phylogenetic correction. Likewise, measures of climatic divergence, including latitudinal distance, were only marginally correlated with cross failure, flower number and fruit set, but again, these relationships were absent with phylogenetic correction. Glucosinolate profile distance was also marginally associated with increased cross failure, but not when correcting for phylogeny. Leaf shape was the only ecological character consistently associated with reproductive isolation. For both cross failure and seed germinability, leaf shape divergence was positively correlated with increased reproductive isolation; however, these results were not robust to phylogenetic correction.

It is possible than that adaptive divergence in leaf shape may have accelerated the evolution of reproductive isolation at earlier stages of hybrid survival. Leaf lobedness has functional implications for water transportation and temperature regulation (Sack and Holbrook 2006), and could therefore have been a target of divergent adaptation to different latitudes or habitats (coastal vs.

desert). However, further investigation is required to ascertain both the adaptive significance of these leaf traits as well as how they contribute to intrinsic post-zygotic RI in this system. Selection and reciprocal transplant experiments would provide insight into the functional importance of leaf lobedness across different habitats, while a more focused crossing study with F_2 hybrids could provide insight into whether the leaf traits and hybrid inviability were linked (Macnair and Christie 1983; Lowry and Willis 2010).

We found little evidence that ecological divergence in climate has contributed to the evolution of intrinsic post-zygotic RI. One explanation could be that the ecological divergence between species has actually been weak, as evidenced by many of the species' large latitudinal ranges. For many species, the level of intra-species climatic variation was similar to the level of inter-specific climatic variation. However, divergent adaptation to climate apparently has occurred at the subspecific level (see Chapter 3), suggesting that ecological differences in climate do reflect some level of adaptive divergence. Climate does appear to exert selection in the genus even though it does not appear to have driven the evolution of reproductive isolation in this group. It is possible that adaptation to more locally variable edaphic conditions, such as water availability, or community characteristics, such as presence of specific herbivores, may have contributed to the evolution of intrinsic post-zygotic RI

within the group. Further investigation of the adaptive importance of these alternative factors is necessary.

An interesting finding is that ecological divergence contributes more strongly to RI expressed at early stages of hybrid inviability than to later stages. One reason for this is that selection on early life stages may be especially pronounced, if only because organisms must survive early stages before they can adapt at later life stages (Donohue et al. 2010). Adaptive divergence later in life may not accrue unless early stages adapt first. This may be especially true for colonizing species, such as *Cakile*, in which the colonizing propagules are the earliest life stage: seeds. While these adaptive dynamics have been investigated to a limited degree within species (Donohue 2013, in review), they have not been interpreted within the context of speciation and the contribution of divergent adaptation to the evolution of RI. In addition, there are more caveats to the interpretation of our fitness data, versus or survival data. . The use of flower number and fruit set as measures of fecundity is complicated by the fact that taxa vary in mating system (i.e., self-compatible vs. self-incompatible). These issues, in turn, can also complicate the interpretation of relative F_1 fitness, depending on how mating system alleles interact in the hybrids. For instance, self-compatibility in the hybrids may result in higher fitness of hybrids than of the self-incompatible parent.

In conclusion, we found significant intrinsic post-zygotic reproductive isolation in *Cakile* that is strongest in earlier stages of hybrid development and that has some association with ecological divergence. However, divergent adaptation to climate does not appear to have contributed strongly to the evolution of these components of reproductive isolation. Linkage (or pleiotropy) between loci under divergent selection and loci that contribute to intrinsic incompatibilities does not appear to be a significant contributor to speciation dynamics in this group. How divergent selection contributes to post-zygotic reproductive isolation as a consequence of disruption of adaptation itself, however, remains to be tested.

4.5 Addendum

There is the possibility for additional analyzes with these data that were not included in this paper, but address two major questions in speciation biology: 1) the degree of cross asymmetry in the evolution of reproductive isolation, and 2) the influence mating system on the evolution of reproductive isolation. I intend to prepare another paper from this data set that addresses these questions.

Cross asymmetry in reproductive isolation is commonly observed in plants, especially in post-mating barriers (Tiffin et al. 2001). This pattern is generally attributed to cytonuclear or gametophyte–sporophyte conflicts (Fishman and

Willis 2006; Leppälä and Savolainen 2011; Caruso et al. 2012), but also could result from differences in parental silencing (Josefsson et al. 2006; Turelli and Moyle 2007; Lowry et al. 2008a; Crespi and Nosil 2013). What is less well understood is how ecological divergence might play a role in promoting the evolution of these asymmetries. The models which predict the evolution of asymmetries (Turelli and Moyle 2007), however, operate under similar processes as the models which predict the evolution of genic incompatibilities. Therefore, similar predictions could be made about the role of divergent adaptation in promoting the evolution of asymmetries i.e., increased genetic turnover and greater probability of linkage pulling along incompatibilities.

We can test this prediction with this dataset by comparing the magnitude of asymmetries in reproductive isolation with the magnitude of ecological distance. The reproductive isolation data for this study was generated from fully reciprocal crosses between all 18 parental taxa. This design allows us to calculate how the level of reproductive isolation at any given barrier differs between the maternal and paternal cross i.e., the magnitude of asymmetry. We can then test if this metric of asymmetry correlates with ecological distance using the same methods described above. A significant positive correlation would suggest that divergent adaptation has accelerated the evolution of either inter-genomic or inter-generational conflict.

Second, we address the role of mating system (i.e., self-compatible [SC] vs. self-incompatible [SI]) in the evolution reproductive isolation. We can test the effect of mating system with this dataset because 5 of the 18 taxa are identified as self-compatible species, while the other species exhibit self-incompatible mating systems. On one hand, this question is similar to the question of asymmetrical reproductive isolation. Mating system differences are predicted to influence post-mating reproductive isolation (Brandvain and Haig 2005; Crespi and Nosil 2013), such that SI species are predicted to be able to pollinate, but not be pollinated by, SC species. This dataset allows us to uniquely test the specific effect of mating system on the magnitude of RI between parents with similar versus different mating systems. In particular, we can test whether SC species are less able to pollinate SI species than vice versus because of strong parental conflicts in SI species (Brandvain and Haig 2005). Furthermore, this dataset allows us to test the effect of mating system on reproductive isolation in the context of ecological divergence. More specifically, we can test if the degree of ecological divergence between the parental taxa influences the degree of SC vs. SI conflict.

5. Reproductive isolation in the genus *Cakile* in association with latitudinal divergence

5.1 Introduction

The importance of divergent selection in speciation has been seriously questioned (Coyne and Orr 2004). The contribution of divergent selection to the evolution of reproductive barriers between taxa is not in itself controversial, but the degree to which divergent selection accelerates the evolution of different components of reproductive isolation, particularly post-mating and intrinsic reproductive isolation, remains poorly understood (Schemske 2010).

Selection can be either divergent, favoring different traits in different environments, or parallel, favoring similar traits in different populations that inhabit similar environments (McKinnon et al. 2004; Schluter 2009; Conte and Arnegard 2012). Studies concerned with the role of selection in speciation typically focus on divergent selection. This is because divergent selection is thought to be more likely to result in greater genetic turnover that promotes the evolution of reproductive isolation. In other words, divergent selection is likely to fix novel traits or loci that either directly or indirectly result in reproductive isolation (Coyne and Orr 2004; Rundle and Nosil 2005; Schluter and Conte 2009). In the simplest scenario, divergent selection acts on a trait or locus that directly causes reproductive isolation. Alternatively, divergent selection may act on a

trait or locus that is pleiotropic or physically linked with another trait or locus that causes reproductive isolation.

Whether divergent selection is important to the evolution of reproductive isolation depends on the reproductive barrier in question. Divergent selection, in contrast to parallel selection, is expected to accelerate the evolution of reproductive isolation among barriers that are likely to experience direct natural or sexual selection (Weissing et al. 2011). These barriers are typically pre-zygotic, in that they prevent the parents from mating. For example, natural selection on floral traits imposed by different pollinator communities can lead to assortative mating (Johnson and Steiner 1997; Kay and Sargent 2009; Moe et al. 2012) or adaptation to different habitats can result in immigrant inviability and habitat sorting (Nosil 2007; Lowry et al. 2008b; Feder et al. 2012). Divergent selection typically affects post-mating barriers by disrupting adaptive complexes in the hybrid offspring, making them maladapted to either parental environment (Schemske 1999; Gow et al. 2007; Lowry and Willis 2010).

In contrast, the role of divergent selection in the evolution of barriers that are not under direct selection is less clear (Schemske 2010). In particular, whether divergent selection accelerates the evolution of post-mating pre-zygotic barriers such as pollen-pistil interactions or intrinsic post-zygotic barriers that reduce hybrid fitness, such as genic incompatibilities, is poorly understood and,

consequently, controversial (Coyne and Orr 2004; Schluter and Conte 2009; Schemske 2010; Langerhans and Riesch 2013).

Whether divergent selection will accelerate the evolution of non-ecological barriers, that is, intrinsic reproductive isolating barriers, depends on the number of alleles under selection, as well as their origin (novel versus ancestral) and the order in which they are fixed (Schluter and Conte 2009). Theory predicts that populations adapting in parallel to similar selective environments will fix as many novel alleles as populations undergoing divergent adaptation to different environments, provided that the strength of selection is the same and mutations occur randomly and without replacement (Orr and Turelli 2001; Unckless and Orr 2009). This is known as a "mutation-order" model and will lead to the same amount of genetic turnover in both selective regimes (Schluter and Conte 2009; Langerhans and Riesch 2013). Under a mutation-order model, the same number of intrinsic genetic barriers is predicted to arise under both parallel and divergent selection.

However, divergent selection can accelerate the relative rate of evolution of intrinsic barriers in two ways. First, if divergent selection is stronger than parallel selection, it will increase the overall degree of genetic turnover between two species and thereby increase the likelihood that alleles contributing to reproductive isolation become fixed either directly or, more probably, via linkage

(Schluter 2009; Schluter and Conte 2009; Langerhans and Riesch 2013). Second, even if the strength of selection is the same, the relative rate of genetic turnover among divergent populations might be greater if populations under parallel selection often fix the same alleles. For instance, populations undergoing parallel selection might be predisposed to fix similar alleles already present in the standing variation of their original population whereas those under divergent selection would fix different alleles (Barrett and Schluter 2008; Conte and Arnegard 2012). In short, it is theoretically possible that divergent selection can increase the degree of intrinsic reproductive isolation involving non-ecological barriers relative to parallel selection; therefore, whether divergent selection accelerates the evolution of intrinsic reproductive isolation is largely an empirical question.

Few studies have examined how divergent selection has influenced post-mating reproductive isolation. For post-mating pre-zygotic barriers in plants, the potential importance of divergent selection is clearer: morphological changes in floral traits, changes in self-compatibility, or pollen competition due to divergent selection can have direct impacts on pollen-pistil interactions. Indeed, there is greater empirical evidence that divergent adaptation has played a role in the evolution post-mating pre-zygotic barriers in plants. Putative associations between adaptive divergence and increased incompatible pollen-pistil

interactions between species pairs have been identified in *Faramea, Phlox* and *Costus* (Travers 1999; Kay 2006; Ruane and Donohue 2007). While theory predicts that is possible, whether divergent selection accelerates the evolution of intrinsic incompatibilities is less clear. There is no *a priori* reason to assume that loci involved with intrinsic genic incompatibilities are pleiotropic with or linked to traits under divergent selection. However, some empirical evidence exists that certain hybrid incompatibilities are linked to traits under selection. In *Mimulus guttatus*, a hybrid lethality locus was found to be physically linked to a locus associated with divergent adaptation to copper tolerance (Macnair and Christie 1983; Wright et al. 2013). Similarly, in *Arabidopsis thaliana*, hybrid necrosis has been shown to be pleiotropic with autoimmune responses that confer disease resistance (Bomblies and Weigel 2007), although variation at this locus may be maintained by balancing selection within populations rather than divergence among populations. Finally, within the species *Collinsia sparsiflora*, there is evidence for the evolution of hybrid infertility between populations that have undergone divergent adaptation to serpentine soils (Moyle et al. 2012).

Empirical evidence that divergent selection contributes to the evolution of post-mating reproductive isolation comes primarily from studies of species pairs. With the exception of the *Collinsia* study, these studies lack a controlled comparison between divergent versus parallel selection. To address this concern

directly, one would ideally use a focal-cross design (Figure 13). A focal-cross design is designed to compare the degree of reproductive isolation between a focal taxon and a related pair of sister taxa, in which one sister has undergone divergent adaptation and the other sister has undergone parallel adaptation, relative to focal taxon. By using sister taxa, one can control for divergence time (i.e., genetic distance).

The genus *Cakile* Mill. (Brassicaceae) offers an ideal system in which to test whether divergent selection accelerates the evolution of intrinsic reproductive barriers using a focal-cross design. All coastal *Cakile* species inhabit the maritime strand, which is broadly similar across its range in terms of edaphic factors, drought stress, disturbance, resource availability, competition, and dispersal availability (Rodman 1974). However, there have been repeated diversification events among sister taxa across latitudes that are both parallel (e.g., Caribbean vs. Mediterranean coastal environments at similar latitude) and divergent (e.g., subspecific diversification across a wide latitudinal range in both Europe and North America).

Two *Cakile* species, *C. maritima* in Europe and *C. edentula* in North America, inhabit a wide latitudinal range and at least *C. edentula* appears to have adapted to northern climates during range expansion northward following de-glaciation (see Chapter 3). Evidence for adaptive differences across a climatic

gradient among *Cakile* species includes a genetic differential between latitudinal (northern vs. southern) races (see Chapter 2) and differences in leaf functional traits (lobedness) and glucosinolate content correlated with climatic divergence (Appendix A). An experiment conducted in growth chambers that imposed different climatic scenarios representative of different latitudes showed evidence of sub-specific differentiation in flowering time, and fitness-related traits such as flower and fruit number (see Chapter 3), consistent with adaptation of the northern subspecies of *C. edentula* to northern climates experienced during range expansion in North America. Therefore, divergent selection appears to have contributed to adaptive divergence among subspecies across latitude.

Such subspecific adaptive divergence provides an excellent opportunity to test whether divergent selection accelerates the evolution of reproductive isolation. Specifically, it provides taxa that have adapted to similar versus different latitudes from a focal species, while both taxa are equally genetically related to it. *Cakile* is well suited for a focal-cross design because it not only provides sister taxa that have likely undergone divergent and parallel selection, but it also provides these taxa across a range of genetic distances (see Chapter 3, Appendix A).

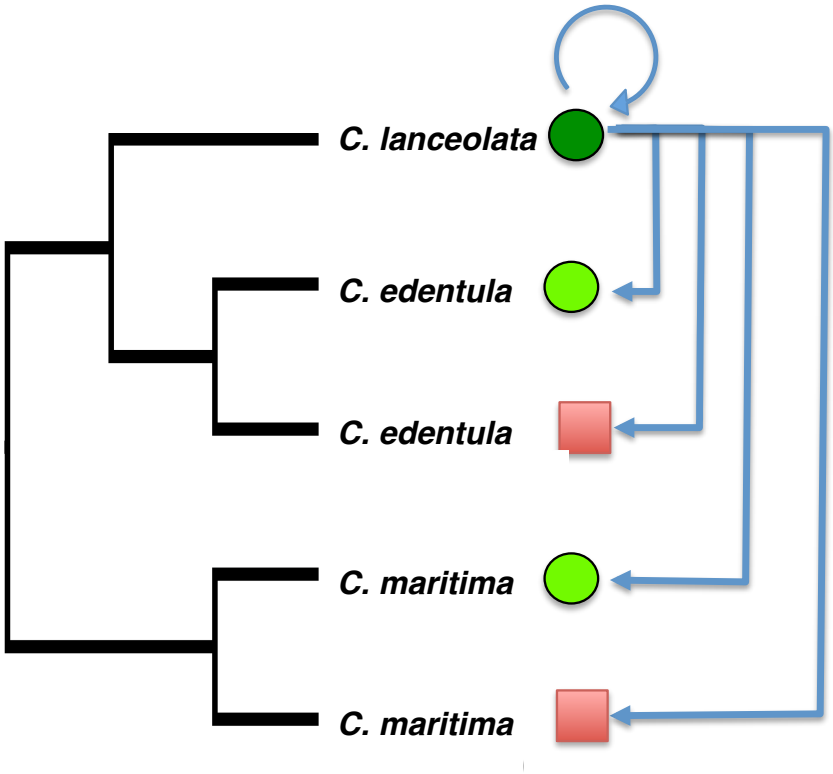


Figure 13. Schematic of Focal-Taxon Cross Design. Focal taxon (*C. lanceolata)* is shown as a dark green circle. Crosses are indicated by blue lines. Taxa from northern latitudes are shown as red squares. Taxa from southern latitudes are shown as green circles and include the focal taxon. Cross design accounts for divergence time by comparing sister taxa that are equally related to the focal taxon.

Here, we measure the magnitude of several intrinsic post-mating barriers using a focal-cross design of ecologically similar versus divergent taxa in the genus *Cakile*. Specifically, we test a) whether multiple measures of intrinsic

reproductive isolation are higher in more distantly related taxa, and b) whether intrinsic reproductive isolation is higher between crosses of taxa that inhabit different versus similar latitude, controlling for genetic distance.

5.2 Material and Methods

To test for the effects of ecological divergence on the evolution of reproductive isolation, we used a focal-cross design (Figure 13). The focal taxon, *C. lanceolata*, was collected from two populations in the Bahamas. We performed a set of reciprocal crosses between our focal taxon and a northern and southern population of *C. maritima* and *C. edentula*. The northern *C. edentula* population was from Point Judith, Rhode Island (USA), and the southern *C. edentula* population was from Tybee Island, Georgia (USA). The northern *C. maritima* population was from Gdansk, Poland, and the southern *C. maritima* population was from Tarifa, Spain (kindly provided by Dr. Sulisława Borzyszkowska and Dr. Rafael Rubio de Casas, respectively). This resulted in two sets of north-south and south-south crosses. These crosses were compared to intra-specific crosses with *C. lanceolata*.

Both molecular and experimental evidence suggests that populations from different latitudes have adaptively diverged. Based on taxonomic and molecular data, the genus *Cakile* can be delineated into three major geographic groups: European (*C. maritima*), North American (*C. edentula*), and Caribbean (*C.*

lanceolata sensu lato). The Caribbean clade is sister to the North American clade, while the European clade acts as an out-group (see Chapter 2). Within the European and North American clades, there are genetically and morphologically distinct northern and southern populations. The North American taxa consist of *C. edentula edentula* (North) and *C. edentula harperii* (South). The European taxa consist of *C. maritima baltica* (North) and *C. maritima maritima* (South). Evidence for genetic divergence in North America supports distinct northern and southern populations (Gormally and Donovan 2011). Similarly, there is evidence for genetic divergence among the European populations, with the northern populations exhibiting patterns of recent range expansion and population growth (Clausing et al. 2000; Westberg and Kadereit 2009). In addition to molecular evidence, North American populations appear to have undergone adaptive range expansion into the north, as discussed above (see Chapter 3, Appendix A).

For each taxon, 2-3 replicates from 1-6 families (median = 4) were grown at Duke University under standard greenhouse conditions (12/12 hours day/night; 25 C). Experimental reciprocal crosses were performed between randomly selected families from each pair of taxa plus focal taxa (median = 12 crosses per family pair). To prevent self-pollination, flowers selected for crossing were emasculated in-bud prior to anthesis, the night before the cross. Crosses were

performed the following morning by hand. Crosses between the same individuals were performed on different days to minimize the impact of differential resource allocation across time on cross success. From these crosses, reproductive isolation via pollen-pistil interactions (PPI) and F_1 fertility were measured as described below.

We measured three distinct phases of pollen-pistil interactions using the protocol from Ruane and Donohue (2007) and Pittman and Levin (1986): adhesion (number of pollen grains attached to stigma), pollen germination (total number of pollen tubes), and pollen tube growth rate (number of pollen tubes past base of style after 6 hours). Flowers were collected 6 hours after pollination and preserved in 70% EtOH. Flowers were then stained in 0.005% toluidine blue and fixed in 0.1% decolorized aniline blue. After fixation, pistils were dissected out and observed under a UV florescence microscope. Pollen viability of hybrid offspring was measured using a 1% thiazolyl blue + 5% sucrose solution. Pollen was collected from recently dehisced anthers and stained within 1-2 hours of collection. Pollen viability was assessed by eye. Up to 100 pollen grains were counted and scored as viable or non-viable based on pollen color.

Ovule number of hybrid offspring was measured on pistils stained with 1% thiazolyl blue + 5% sucrose solution. The 0.005% toluidine blue highlighted

vessels making it easier to distinguish between ovules and other tissue. Pistils were dissected by hand and ovule numbers were recorded.

For each stage of reproductive isolation, reproductive isolation was assessed relative to intraspecific success: reproductive isolation = 1 – (average success of interspecific cross/average success of intraspecific cross). In certain incidences, interspecific success was greater than intraspecific success, resulting in a negative RI value (heterosis). These values were not removed. All measures of reproductive isolation can, therefore, range between -∞ to 1 (complete reproductive isolation).

To test for statistical differences in PPI and F_1 fertility associated with ecological divergence, we performed a two-way analysis of variance (ANOVA) accounting for species differences (*C. edentula* vs. *C. maritima*), ecological differences (north vs. south), and their interaction. Replicates were averages across family. All factors were treated as fixed factors. Pollen adhesion and germination were exponentially transformed to meet the assumptions of normality. Pollen growth rate and F_1 pollen viability met the assumptions of normality without transformation. Residuals from the analysis of F_1 ovule number were not normally distributed, however, and no transformation significantly improved the normality. Results for ovule number represent those from untransformed data. Because of significant or marginally significant

interactions between species and latitude (north vs. south), we also tested for differences among northern and southern populations within each species using one-way ANOVA.

5.3 Results

Mean values of measures of pollen-pistil interactions and fertility of F_1 crosses are shown in Table 26. Significant reproductive isolation was detected between *C. lanceolata* and both *C. edentula* and *C. maritima* for several reproductive barriers (Tables 27, Figures 14 and 15). Reproductive isolation via pollen-pistil interactions (pollen adhesion, germination, and tube growth rates) were of smaller magnitude than reproductive isolation via pollen viability and ovule number (Table 27).

No measure of pollen-pistil interaction differed significantly between *C. maritima* and *C. edentula* (Tables 27 and Table 28, Figure 14). However, *C. maritima* showed significantly stronger RI than *C. edentula* with respect to pollen viability, as indicated by the significant species effect (Table 28, Figure 14). Hybrids with *C. maritima* had three-fold greater pollen inviability than hybrids with *C. edentula.* F_1 ovule number did not differ significantly between species (Table 27, Figure 14). Thus *C. edentula* and *C. maritima* had comparable levels of reproductive isolation with *C. lanceolata* with respect to PPIs and ovule number,

but the more distantly related *C. maritima* had greater RI with *C. lanceolata* with respect to hybrid pollen viability (Table 27, Figure 14).

No main effect of latitude was detected on all three stages of pollen-pistil interactions (Table 28). Rather, latitudinal effects appear to be species-dependent, as indicated by significant or marginally significant interactions between species and latitude (Table 28, Figure 15). Northern *C. maritima* had significantly higher adhesion rates (i.e., lower RI at this stage) than southern *C. maritima*, counter to the expectation that hybrids from crosses of similar latitude would have lower RI. Northern *C. edentula* had marginally significant slower pollen tube growth rates (i.e., higher RI at this stage) than southern *C. edentula* (Table 28, Figure 15).

No significant main effect of latitude was detected on F_1 pollen viability, nor was there a significant interaction between latitude and species on F_1 pollen viability (Table 28, Figure 15). There was, however, a marginally significant main effect of latitude on F_1 ovule number (Table 28, Figure 15). North-south hybrids, on average, had more ovules than south-south hybrids, contrary to expectation. There was no significant interaction between latitude and species for F_1 ovule number (Table 28).

Table 26. F_1 means of pollen-pistil interactions, male fertility, and female fertility. "Cross type" indicates what *C. lanceolata* was crossed with. Pollen-pistil interactions include: number of pollen grains adhered to the stigma (pollen adhesion), number of pollen grains that produced a pollen tube (pollen germination), and proportion of pollen grains that passed the style after 6 hours (pollen tube growth rate). Male fertility was measured as the proportion of inviable pollen grain based on thiazolyl stain (pollen inviability). Female fertility was measured as the number of ovules present in the ovary (ovule number).

	Pollen Adhesion			Pollen Germination			Pollen Tube Growth Rate			Pollen Inviability			Ovule Number		
Cross Type	**n**	**mean**	**SE**	**n**	**mean**	**SE**	**n**	**mean**	**SE**	**n**	**mean**	**SE**	**n**	**mean**	**SE**
C. edentula (N)	18	33.67	8.50	20	16.15	1.35	19	0.59	0.04	191	0.30	0.02	28	2.96	0.16
C. edentula (S)	43	29.07	4.45	44	17.18	0.89	43	0.69	0.02	237	0.26	0.02	35	2.86	0.14
C. lanceolta (Focal)	20	29.55	6.25	20	19.05	1.22	19	0.64	0.04	78	0.21	0.02	13	3.85	0.10
C. maritima (N)	49	31.80	3.59	50	16.34	0.70	50	0.71	0.02	152	0.43	0.03	43	3.09	0.12
C. maritima (S)	47	14.98	2.92	47	15.38	0.69	46	0.74	0.03	272	0.37	0.02	46	2.72	0.12

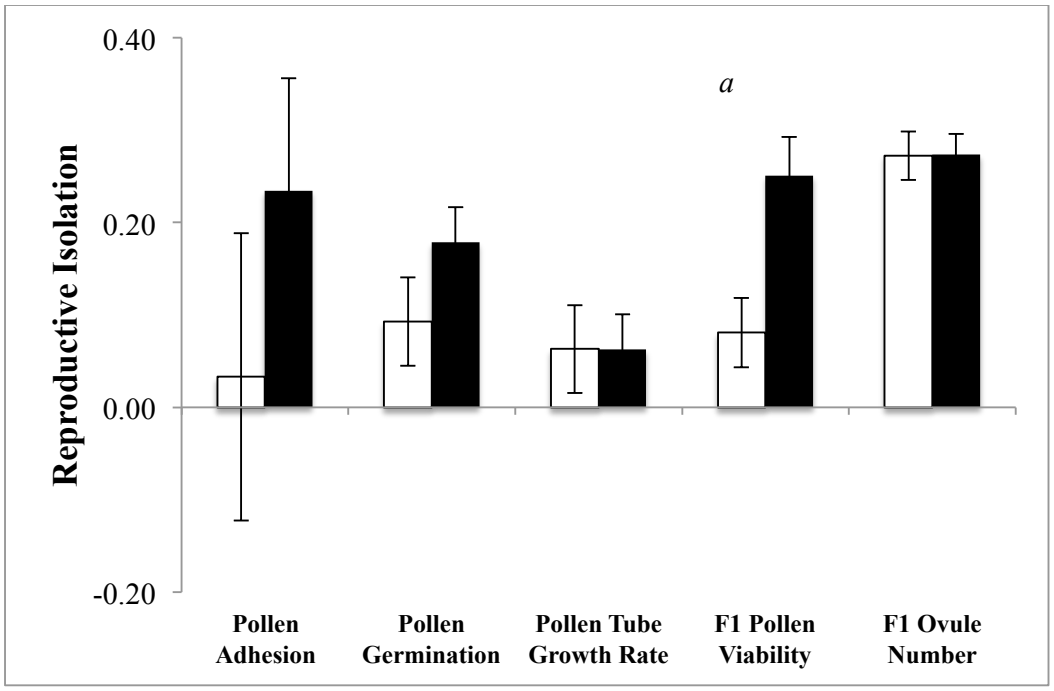


Figure 14. Mean reproductive isolation between *Cakile lanceolata* and *C. edentula* (white) and *C. maritima* (black). Error bars indicate standard errors. Pollen-pistil interactions include: pollen adhesion (number of pollen grains on stigma), pollen germination (number of pollen tubes germinated), and pollen tube growth rate (number pollen tubes to reach the end of style after 6 hours). Measures of F_1 fertility include pollen viability and ovule number. *a* indicates $P < 0.05$ based on a two-way ANOVA and Tukey HSD adjusted comparison of means.

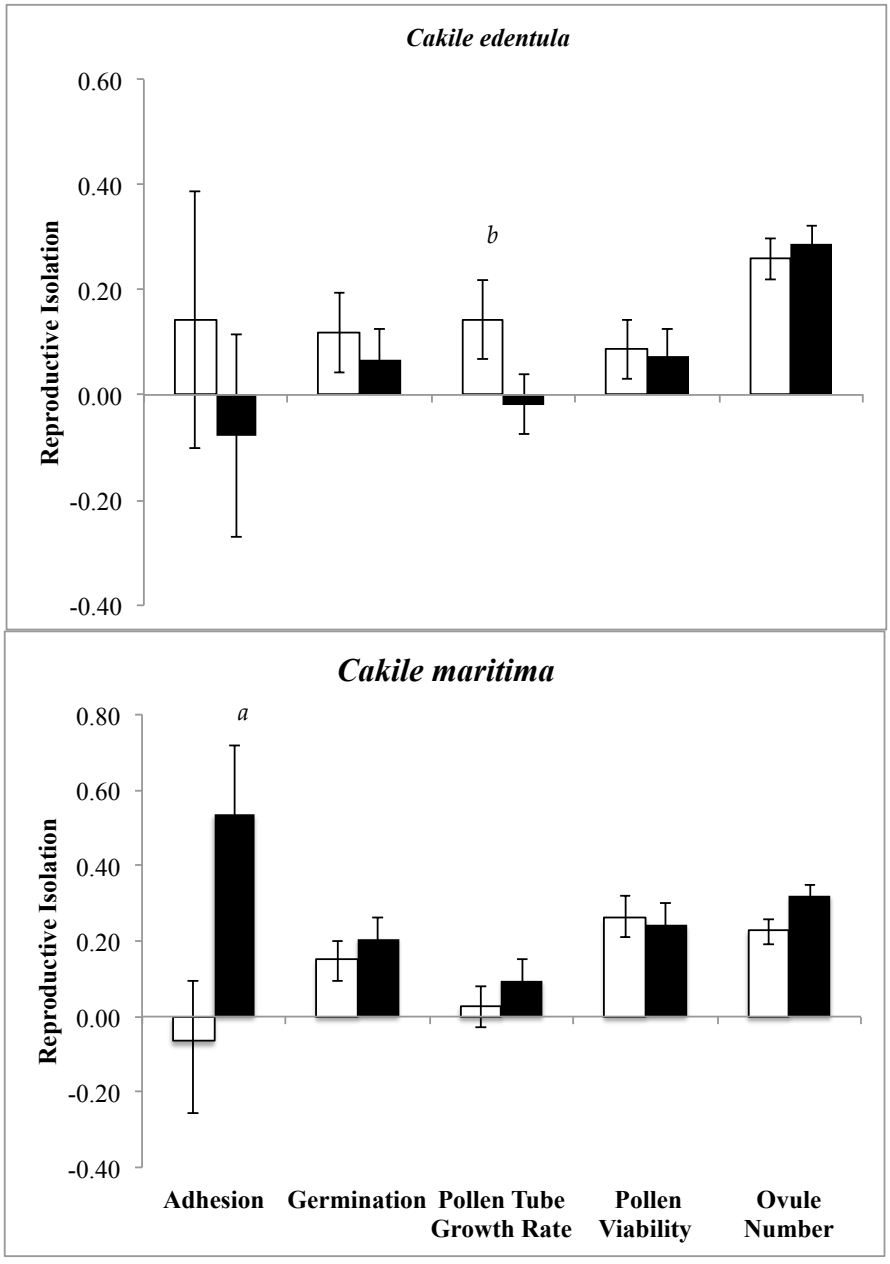


Figure 15. Mean reproductive isolation between *Cakile lanceolata* (southern) and northern populations (white) or southern (black) populations of *C. edentula* (upper) and *C. maritima* (lower). Error bars indicate standard errors. Pollen-pistil interactions include: pollen adhesion (number of pollen grains on stigma), pollen germination (number of pollen tubes germinated), and pollen tube growth rate (number pollen tubes to reach the end of style after 6 hours). Measures of F_1 fertility include pollen viability and ovule number. *a* indicates $P < 0.05$, *b* indicates $P < 0.10$, based two-way ANOVA and Tukey HSD adjusted comparison of means.

Table 27. Least-square means of reproductive isolation between different taxa growing at different latitudes. Reproductive isolation is measured between the focal taxon *Cakile lanceolata* and the species *C. edentula* (North American) and *C. maritima* (European). North and south indicate the latitudinal origin of the species population. Significant effects are in bold ($P < 0.05$). Marginally significant effects are italicized ($P < 0.10$). Boldface indicates a significant difference in a two-way ANOVA in an intraspecific comparison of latitude.

	Adhesion	SE	Germination	SE	Pollen Tube Growth Rate	SE	Pollen Viability	SE	Ovule Number	SE
C. edentula	0.03	0.16	0.09	0.05	0.06	0.05	**0.08**	**0.04**	0.27	0.03
C. maritima	0.23	0.12	0.18	0.04	0.06	0.04	**0.25**	**0.04**	0.27	0.02
North	0.04	0.15	0.13	0.05	0.09	0.05	0.17	0.04	*0.24*	*0.03*
South	0.23	0.13	0.14	0.04	0.04	0.04	0.16	0.04	*0.30*	*0.02*
C. edentula North	0.14	0.24	0.12	0.08	*0.14*	*0.08*	0.09	0.06	0.26	0.04
C. edentula South	-0.08	0.19	0.07	0.06	*-0.02*	*0.06*	0.07	0.05	0.29	0.04
C. maritima North	**-0.07**	**0.16**	0.15	0.05	0.03	0.05	0.26	0.06	0.23	0.03
C. maritima South	**0.53**	**0.18**	0.21	0.06	0.10	0.06	0.24	0.06	0.32	0.03

Table 28. ANOVA results for reproductive isolation between different taxa growing at different latitudes (see Table 1). Significant effects are in bold ($P < 0.05$). Marginally significant effects are italicized ($P < 0.10$).

	Pollen-Pistil Interactions						Hybrid Fertility			
	Adhesion		Germination		Rate		Pollen		Ovule	
Effect	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Latitude	1.74	0.1918	0.00	0.9880	0.17	0.6844	0.30	0.5878	*3.83*	*0.0522*
Species	1.51	0.2234	2.29	0.1349	0.04	0.8340	**9.07**	**0.0035**	0.01	0.9063
Lat X Species	**4.31**	**0.0419**	0.74	0.3928	*3.53*	*0.0648*	0.00	0.9502	0.96	0.3300

5.4 Discussion

We found little evidence that ecological divergence contributes to post-mating pre-zygotic and intrinsic post-zygotic RI in the genus *Cakile*. While there were significant levels of reproductive isolation for pollen-pistil interactions and hybrid fertility (F_1 pollen viability, F_1 ovule number), they depended more on genetic distance than ecological differences between taxa. The one exception to this was that pollen tubes grew more slowly in crosses between latitudinally divergent taxa (northern *C. edentula* X southern *C. lanceolata*) than between latitudinally non-divergent crosses (southern *C. edentula* X southern *C. lanceolata*). The only other differences associated with latitudinal divergence were in the opposite direction from that predicted, with divergent crosses between *C. maritima* and *C. lanceolata* exhibiting weaker reproductive isolation via pollen adhesion and F_1 ovule number than non-divergent crosses.

More genetically distant crosses produced hybrids with lower pollen viability. Other studies have found genetic distance to be positively correlated with pollen-pistil interactions (Moyle et al. 2004; Jewell et al. 2012), and hybrid pollen inviability (Moyle et al. 2004; Martin and Willis 2007; Moyle and Nakazato 2010; Moyle et al. 2012). The result is also consistent with the evolution of other measures of intrinsic post-zygotic reproductive isolation within *Cakile* (see Chapter 4). In a broad study of 18 taxa from the genus *Cakile* and its sister genus

Erucaria, post-zygotic reproductive isolation, particular in early stages of hybrid viability, was found to increase with genetic distance.

Interestingly, in Chapter 4, we found relatively weak levels of reproductive isolation among hybrid fecundity measured as flower and fruit production. This is in contrast to our results here with regard to hybrid fertility. Both male (pollen) and female (ovule number) fertility exhibited relatively high levels of reproductive isolation, similar to levels observed for early stages of hybrid viability in *Cakile.* Thus, while hybrids between *Cakile* taxa may not be limited by flower production or seed set via selfing, these flowers or fruits may, nonetheless, be limited by fertility.

Overall, ecological divergence across latitude did not strongly affect levels of reproductive isolation. Northern *C. edentula* exhibited significantly but modestly higher levels of reproductive isolation with southern *C. lanceolata*, based on pollen-tube growth rates. The only other significant differences between divergent versus non-divergent crosses were for the pollen adhesion rates of *C. maritima* and ovule number of hybrids with northern *C. maritima*, and both were in the opposite direction as predicted: the southern *C. maritima* (less ecologically divergent) had weaker pollen adhesion to the stigma (greater RI) than their northern counterpart, and hybrids with northern *C. maritima* (more divergent) had more ovules.

These results do not support predictions that ecological divergence contributes to reproductive isolation. Instead, to the extent the ecological divergence observed here reflects adaptive divergence, it appears that post-mating intrinsic reproductive barriers in the genus *Cakile* have evolved at similar rates under divergent and parallel selection. This would suggest that either a) loci contributing to reproductive isolation are not linked to loci under divergent selection, or b) taxa under parallel selection in similar environments have adapted by fixing alternative mutations, at a rate similar to divergent selection (i.e., mutation-order model Nosil and Flaxman 2010); likewise, taxa under divergent selection could in principle fix alleles from standing genetic variation at a rate comparable to parallel adaptation (Schluter 2009; Conte and Arnegard 2012).

First, loci associated with post-mating intrinsic reproductive isolation may not be under divergent selection. Genes that regulate pollen-pistil interactions, pollen development, and ovule formation are unlikely to be the direct target of latitudinally divergent natural selection, since it is the stigma and stylar environment that exerts selection on these pollen traits more strongly than does the external ecological environment. However, in specific cases, pollen-pistil interactions have been shown to be dependent on soil (Ruane and Donohue 2007) and climatic conditions (Travers 1999), suggesting some possibility for

adaptation of pollen performance to external ecological conditions. It is possible that pollen-pistil interactions in *Cakile* may be similarly environmentally dependent, and would manifest more strongly if crosses were carried out under the environmental conditions of the parental plants. There is also little evidence that genes regulating pollen-pistil interactions are closely integrated with genes or developmental networks that would be under ecologically divergent selection; for instance, pollen performance (e.g., pollen-tube growth rates) frequently have little effect on seedling performance (Pietarinen and Pasonen 2004; Lankinen et al. 2009), a trait that often experiences strong natural selection (Donohue et al. 2010).

Second, adaptation to parallel environments could lead to just as much genetic turnover as divergent adaptation, and therefore contribute just as strongly to reproductive isolation as does adaptation to divergent environments (Unckless and Orr 2009). Parallel adaptation has been shown to proceed via the fixation of the same alleles from standing variation (Conte and Arnegard 2012). However, there are also numerous examples of parallel adaptation resulting in the fixation of different mutations that confer the same adaptive phenotype, even in populations that are recently divergent from the same source population (Arendt and Reznick 2008; Streisfeld and Rausher 2009; Ralph and Coop 2010). Thus parallel adaptation has the potential to contribute to the evolution of

intrinsic reproductive isolation to a comparable degree as divergent adaptation. Similarly, divergent adaptation could, in principle, occur by selection acting on standing variation as well, leading to a similar result.

While our results provided little evidence that divergent adaptation to latitude contributes to the evolution of post-mating pre-zygotic and intrinsic post-zygotic reproductive isolation, it is possible that divergent selection among taxa imposed by other factors unrelated to latitude may still be important. Our design focused on the contrast of northern and southern populations, which do show latitudinal divergence in important functional traits as well as fitness. However, it is possible that southern populations of each species may be just as divergent from the focal (southern) species as the northern populations are. Other adaptively significant ecological factors may differ with latitude and contribute to reproductive isolation. The most obvious candidates are herbivore and pathogen communities. Only field studies of multiple taxa with different degrees of ecological divergence would be able to resolve this issue.

In conclusion, rates of intrinsic reproductive isolation within the genus *Cakile* do not appear to be accelerated by latitudinal divergence. Adaptations that underlie transitions to divergent latitudinal environments are not likely associated with mechanisms that alter pollen-pistil interactions or hybrid fertility. Instead, intrinsic post-mating reproductive isolation has evolved

primarily in proportion with the degree of divergence time (i.e., genetic distance) irrespective of latitudinal adaptation.

6. Conclusion

My dissertation explored the role of dispersal and adaption in diversification and the formation of new species. More specifically, I was interested in how adaptation at the species level influenced the process of speciation, and subsequently contributed to the patterns of diversification observed at the macro-evolutionary level. I employed a combination of comparative and quantitative genetics methods to address this question.

In Chapter 2, I documented an association between dispersal-related traits and speciation rates, implicating dispersal as an important factor in speciation. First, I found that dispersal-related fruit traits co-evolve with each other and with seed traits in ways that likely enhance long-distance dispersal and establishment ability. Specifically, indehiscence was associated with dispersal-enhancing features such as abscising joints of heterocarpic fruits and with pericarp adornments that increase dispersal ability. Transitions to long-distance dispersal were subsequently associated with fewer but larger seeds per fruit, which likely increases establishment ability after long-distance dispersal. As predicted, establishment ability (i.e., seed size) was associated with greater latitudinal range. The evolution of seed size was also associated with shifts in habitat. The combined effect of these evolutionary transitions in dispersal ability and habitat

was an increase in the rate of diversification within the tribe via increased speciation rates.

These results suggest a duel role of dispersal and adaptation in the diversification of this tribe. The evolution of long-distance dispersal, in association with greater establishment ability, increases the likelihood of isolation by distance and allopatric speciation. Diversification after long-distance dispersal and colonization may be adaptively neutral (Hubbell 2008) and does not necessarily depend on adaptive divergence for diversification of the clade. However, the association between a shift to coast and an increase in diversification rates leaves open the possibility that an adaptive radiation occurred along niche axes newly available in the coastal environment. One obvious axis is climate, as the coastal clade driving the pattern of diversification, *Cakile*, can be found across a wide range of latitudes and climates.

To test whether *Cakile* has undergone divergent adaptation to local climate, in Chapter 3 I investigated whether two *Cakile* species exhibited evidence for adaptive divergence along a seasonal cline. While there is clear genetic and morphological evidence that delineate northern and southern affiliated subspecies in both species, we found mixed evidence for adaptive divergence in response to climate factors. For the North American species *C. edentula*, we found a significant pattern of range expansion, where the northern

subspecies (*C. edentula* ssp. *edentula*) had higher fruit set under northern seasonal conditions, than its southern counterpart (*C. edentula* ssp. *harperii*). This pattern was not reflected by the subspecies of the European *C. maritima*. The failure to find evidence of adaptive divergence in the European species could be a limitation of the experimental design and our ability to accurately assess fitness for an outcrossing species. In general, however, we can conclude that adaptive divergence has played a role, if limited, in the evolution the genus. The extent to which this adaptive divergence played a role in the speciation process itself, is addressed in the last two chapters.

In Chapters 4 and 5, I investigated the role of ecological divergence in the evolution of intrinsic reproductive isolation, both post-mating pre-zygotic and post-zygotic. Both of these studies used a comparative approach to test for associations between ecological distance and reproductive isolation. In Chapter 4, intrinsic post-zygotic reproductive isolation, measured as hybrid viability and fecundity, exhibited classic patterns of evolution, increasing with genetic distance. There was an association between functional trait divergence and reproductive isolation at the earlier stages of hybrid viability. Beyond this, however, there was no association between ecological divergence and reproductive isolation. In Chapter 5, where I used a more directed experiment design to control for genetic distance, I found no support for an association

between ecological divergence and intrinsic post-zygotic reproductive isolation. Furthermore, there was only weak support for an association between ecological divergence and post-mating pre-zygotic reproductive isolation, and then only in one of the two species examined. Thus, we can conclude that, to the extent *Cakile* has undergone adaptive divergence along a climatic and seasonal cline, the loci under divergent selection do not appear to be linked with loci that contribute to pollen-pistil interactions and intrinsic incompatibilities.

Cumulatively, these results suggest that divergent adaptation has played a minimal role in the evolution of intrinsic reproductive isolation in the Brassiceae, and, in particular, the genus *Cakile.* There was evidence for adaptive divergence in the tribe at both the clade level (Chapter 2) and the species level (Chapter 3). Adaptive shifts at the clade level—the shift to the coast—even appear to be associated with increased rates of speciation. Given the observed ecological divergence among these taxa, it is possible that adaptive divergence has contributed to extrinsic reproductive isolation via contributions to ecological isolation and extrinsic post-zygotic isolation via disruption of adaptive in hybrids. However, only field studies of local adaptation can test the contribution of local adaptation and adaptive divergence to the evolution of these extrinsic components of reproductive isolation. With regard to intrinsic reproductive isolation, the focus of this study, I found limited evidence that divergent

adaptation played a role in facilitating its evolution. Rather, the association between the evolution of long-distance dispersal and increased speciation rates at the clade level suggests that dispersal-mediated isolation events are more important to promoting diversification via intrinsic reproductive isolation within the clade and that the evolution of intrinsic barriers among species evolve primarily as function of isolation time, rather adaptive divergence.

Appendix A: Divergence in the genus *Cakile* along latitudinal and climatic gradients

Genetic Divergence. Based on traditional morphological taxonomy, *Cakile* was divided into three major geographic groups: *C. maritima* (Europe), *C. edentula* (North America), and *C. lanceolata* sensu lato (Caribbean). Additionally, there were also recognized *C. consticta* (Florida), *C. geniculata* (Gulf Coast), *C. arctica* (Iceland), and *C. arabica* (Near East Asia) (Figure 16). Within the three major groups, several subspecies were designated based on morphological and geographic distinction. In the case of *C. maritima* and *C. edentula*, these subspecific designations correspond to latitudinal divergence along a north-south gradient.

These major taxonomic distinctions are roughly supported by molecular data (Chapter 2). *C. maritima*, *C. edentula*, and *C. lanceolata* and their affiliated subspecies appear to be distinct clades within the genus, with significant bootstrap and posterior probability support. *C. edentula* and *C. lanceolata* are sister clades, with *C. maritima* as an outgroup. *C. constricta* and *C. geniculata* fall within the larger *C. lanceolata* clade, with weak support for *C. constricta* as a distinct sub-clade. The affinity of *C. arctica* is less clear. While it does not group with *C. maritima,* it does occur as an outgoup with *C. maritima* to *C. edentula C. lanceolata*, suggesting that it is likely derived from *C. maritima*; however, further

work is needed to determine it's true origin. *C. arabica* occurs alternatively as an outgroup to the larger, coastal *Cakile* clade, or, paraphylatically within the genus *Erucaria (*Chapter 2).

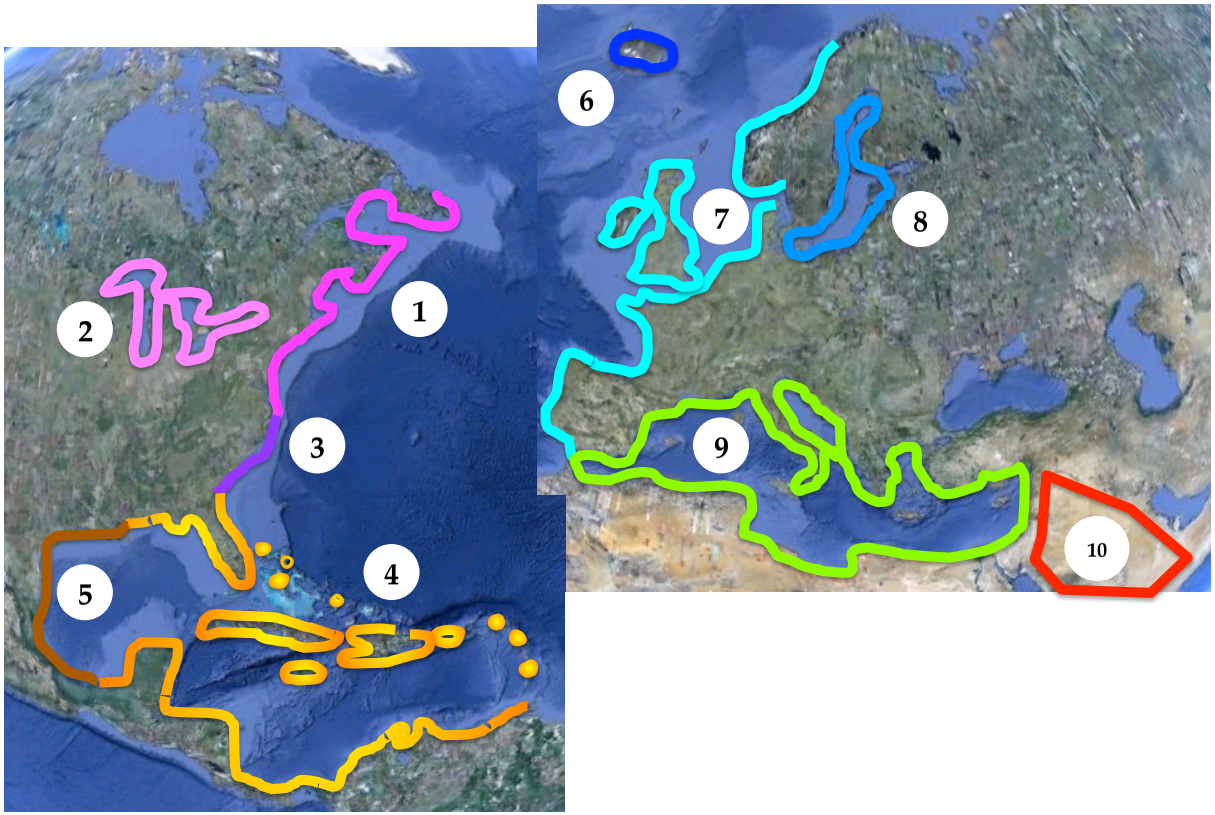
Evidence for genetic divergence along a latitudinal gradient in the European *C. maritima* has been examined in some detail. Traditionally, *C. maritima* has been divided into two taxonomic divisions: a northern subspecies at *C. maritima baltica* and two distinct populations of *C. maritima maritima* in the Mediterranean and Western Europe, distinguished by their ovule development (Rodman 1974). Clausing et al. (2000) examined genetic variation structure across these taxonomic distinctions, from populations sampled across Europe, using random amplified polymorphic DNAs (RAPDs) and intersimple sequence repeats (ISSRs). Cluster analysis of these data identified two major groups within *C. maritima,* a southern Mediterranean clade and a more northern Atlantic clade. These broad biogeographic distinctions were reaffirmed by an additional, expanded studies in 2006 and 2009 (Kadereit and Westberg 2006; Westberg and Kadereit 2009), and roughly corresponding to Rodman's (1974) initial classification; however, the distinction between *C. maritima baltica* and *C. maritima maritima* of the Atlantic was not observed. Rather, it appears that genetic division is the result of geographic isolation due to the Straight of Gibraltar.

There is also evidence for genetic divergence along a latitudinal gradient in the North American *C. edentula*. *C. edentula* has traditionally been divided into three subspecies: *C. edentula edentula* (northern coast of the United States and Canada, NC north to Newfoundland), *C. edentula harperii* (southern coast of the United States, NC south to FL), and *C. edentula lacustris* (Great Lakes). Gormally and Donovan (2011) investigated the genetic variation structure of this group using allozyme loci. They found that the three major taxonomic subspecies were supported by cluster analysis.

Functional Divergence

Glucosinolates. One of the distinguishing characteristics of the family Brassicaceae is the presence of glucosinolate, secondary metabolites that are functionally associated with herbivory resistance (Rask et al. 2000; Ratzka et al. 2002; Taiz and zeiger 2006; Rasmann et al. 2012). Within *Cakile,* glucosinolate levels at different stages of ontogeny have been linked to herbivory levels, with earlier stages of development, particularly in the cotyledons, exhibiting higher levels of glucosinates and lower herbivory rates (Leege et al. 2004). There has also been divergence in glucosinolate profiles within genus that correspond to geographic, taxonomic, and genetic distinctions (Rodman 1976).

Figure 16. Distribution of the genus *Cakile*. 1) *C. edentula* ssp. *edentula* (magenta), 2) *C. edentula* ssp. *lacustris* (pink), 3) *C. edentula* ssp. *harperii* (violet), 4) *C. lanceolata* complex (includes subspecies: *lanceolata, fusiformis, psuedoconstricta,* and *alacranensis*) (yellow/orange), 5) *C. geniculate* (brown), 6) *C. arctica* (blue), 7) *C. maritima,* Atlantic popultion (aqua), 8) *C. maritima* ssp. *baltica* (pale blue), 9) *C. maritima,* Mediterranean population (green), 10), *C. arabica* (red).



Based on analysis of seed glucosinolate profiles, the European *C. maritima* differs from North American *C. edentula* and *C. lanceolata* group (Rodman 1976). Within *C. maritima*, the Atlantic and Mediterranean populations are distinct, mirroring genetic variation. Furthermore, the northern European *C. maritima* spp. *baltica* is distinct from the more southern Atlantic *C. maritima* and Mediterranean *C. maritima*. In the North American *Cakile*, the main distinction is between the southern *C. lanceolata* sensu lato and *C. edentula* spp. *harperii* and the northern *C. edentula* spp. *edentula* and *C. edentula* spp. *lacustris.* In this case, the distinction is primarily latitudinal rather than taxonomic. A comprehensive comparative analysis of the glucosinolate profile principle components for 13 *Cakile* taxa, however, found no broader relationship with latitude (PC1: proportion of variance = 0.75, β_{pglm} = 0.0003, F_{pglm} = 0.93, P_{pglm} = 0.357, $\lambda_{estimate}$ = 1.00; PC2: proportion of variance = 0.20, β_{pglm} = -0.33, F_{pglm} = 0.61, P_{pglm} = 0. 0.451, $\lambda_{estimate}$ = 1.00). There were, however, associations between glucosinolate profiles and bioclimatic variables describing precipitation. PC1 was most strongly associated with precipitation of the wettest month (β_{pglm} = 0.51, F_{pglm} = 7.53, P_{pglm} = 0.0191, $\lambda_{estimate}$ = 0.99), while PC2 was mostly strongly associated with precipitation of coldest quarter (β_{pglm} = 0.09, F_{pglm} = 8.76, P_{pglm} = 0.0130, $\lambda_{estimate}$ = 0.98). These results suggest that glucosinolate composition may have undergone adaptive

divergence in *Cakile* in response to precipitation climate, as well as, latitudinal environment.

Leaf traits. Cakile exhibits a wide range of leaf morphology (Rodman 1976). The variation in leaf morphology is primarily in leaf margin and can range from entire to fully lobate. Leaf lobedness, measured as leaf perimeter/area, is considered a functional trait that corresponds to water use efficiency (hydraulic conductance) and thermoregulation (Brodribb and Holbrook 2003; Sack et al. 2003; Sack and Holbrook 2006). In *Cakile*, leaf lobedness is challenging trait because of the variation observed both across and within species. Leaf lobedness even varies ontogenetically, with more mature plants tending to have more entire margins (Rodman 1974).

To test whether taxa varied in leaf lobedness when controlling for environment and developmental stage, we grew 2 replicates of 4-6 genotypes for 14 taxa under ambient conditions in the Duke University Greenhouse. The youngest, fully developed leaf was collected at first flower for each individual plant. Leaves were kept in deionized water to mitigate the effects of water loss until they were scanned shortly after collection (<2 hours). Leaf perimeter and area were measured in ImageJ v. 1.45 (Schneider et al. 2012). Analysis of variance revealed significant difference among the 14 taxa ($F = 16.2$, $P < 0.001$). This significant variation did not correspond to any obvious geographic or

latitudinal differences, and a comparative analysis of leaf lobedness did not find a significant association with latitude (β_{pglm} = 0.0003, F_{pglm} = 0.53, P_{pglm} = 0.482, $\lambda_{estimate}$ = 0.93). There was, however, a marginally significant association between leaf lobedness and bioclimatic variables associated with precipitation. Precipitation in the driest month had the strongest association and was negatively correlated with leaf lobedness, such that taxa in drier climates tended to have more lobed leaves (β_{pglm} = -0.0003, F_{pglm} = 4.07, P_{pglm} = 0.0687, $\lambda_{estimate}$ = 0.92). This result, while tentative, suggests that leaf shape may have undergone adaptive divergence in *Cakile* in response to water availability, as predicted by its functional association.

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Biography

Charles George Willis was born on March 31st, 1983 in Sioux Falls, South Dakota. He attended the University of Minnesota from 2001 to 2005 where he received a B.S. in Ecology, Evolution, and Behavior and a minor in History. His undergraduate honors thesis work resulted in three peer-reviewed articles: "Phylogenetic community structure in Minnesota oak savanna is influenced by spatial extent and environmental variation", "Antagonistic multilevel selection on size and architecture in variable density settings", and "Genetic variation in tolerance of competition and neighbour suppression in *Arabidopsis thaliana*." From 2006 to 2009, Charles attended Harvard University where he received a M.A. in Organismic & Evolutionary Biology under Dr. Charles Davis. At Harvard he published four peer-reviewed articles: "Phylogenetic patterns of species loss in Thoreau's woods are driven by climate change", "Favorable climate change response explains non-native species' success in Thoreau's woods", "The importance of phylogeny to the study of phenological response to global climate change", and "WALDEN: multi-surface multi-touch simulation of climate change and species loss in Thoreau's woods." Since 2009, Charles has attended Duke University for graduate school in Biology. During his time at Duke, Charles received a Doctoral Dissertation Improvement Grant from the National Science Foundation. He also published one book chapter and one peer-

reviewed article, respectively: “Plant dispersal phenotypes: a seed perspective of maternal habitat selection” and “Germination, postgermination adaptation, and species ecological ranges.” Upon completing his Ph.D. at Duke, Charles will start a post-doctoral fellowship at the Harvard University Center for the Environment.